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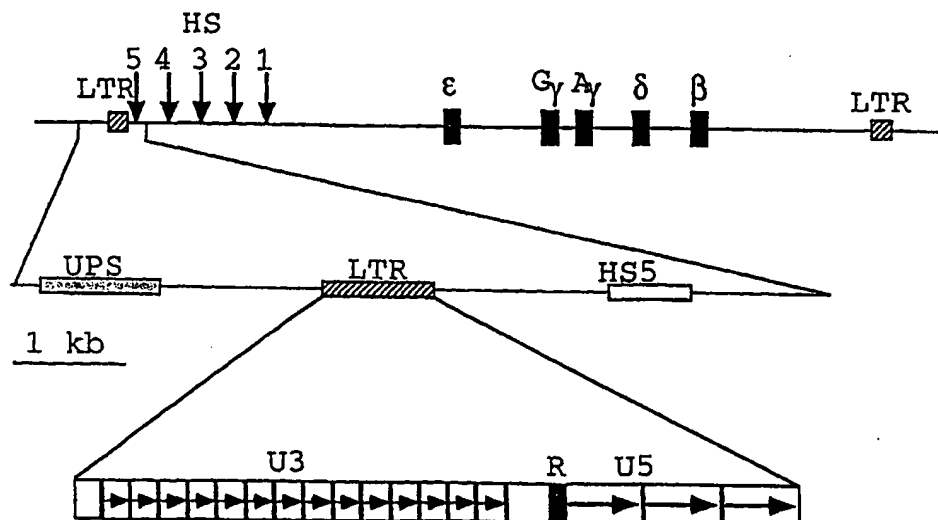
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(54) Title: LONG TERMINAL REPEAT, ENHANCER, AND INSULATOR SEQUENCES FOR USE IN RECOMBINANT VECTORS



(57) Abstract

Disclosed are an enhancer, insulator, and promoter from the HS5 region in the 5' boundary area of the locus control region of human-like globin genes. These transcription control sequences can be used to control expression of any desired gene of interest and can be used in any vector for this purpose. The control sequences are derived from the area in and around the U3 region of a solitary endogenous retrovirus (ERV-9) long terminal repeat (LTR). Also disclosed are methods of expressing any gene of interest. For this purpose, the control sequences can be operably linked to the gene of interest (and operably linked to each other). The disclosed enhancers, insulators, and promoters can also be used with any other control sequences. Preferably, the control sequences are used in vectors to obtain expression of a gene of interest in a cell, including cells in animals.

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LONG TERMINAL REPEAT, ENHANCER, AND INSULATOR SEQUENCES FOR USE IN RECOMBINANT VECTORS

BACKGROUND OF THE INVENTION

The human endogenous retroviruses (HERVs) were inserted into the
5 germ cells of primates millions of years ago and have remained as an integral
part of the primate genomes during evolution. In addition to the proviruses,
solo LTRs are also dispersed throughout the human genome (Wilkinson et al,
1994; Lower et al, 1996). The solo LTRs contain the U3, R and U5 regions
(Temin, 1982) but no internal gag, pol and env genes. Together, the HERVs
10 and the solo LTRs comprise approximately 5% of the human genome and
belong to the category of middle repetitive DNAs characterized as
retrotransposons (A.F. Smit, 1996; Henikoff et al, 1997).

The ERV-9 proviruses, containing 30-50 members, constitute one of
many families of the HERVs (Wilkinson et al, 1993; Lower et al, 1996). In
15 addition to the proviruses, solo ERV-9 LTRs with a copy number of 3000-
4000 have been found in the human genome (Henthorn et al, 1986; La
Mantia, 1991; Schlessiger, 1992). The ERV-9 retrotransposons were
inserted into the primate genome probably as early as ten million years ago
(Di Cristofano et al, 1995). The retrotransposons have been suggested to be
20 selfish DNAs irrelevant to the cellular functions of the hosts (Dolittle and
Sapienza, 1980). However, recent findings indicate that the enhancer and
promoter elements in the U3 region of the LTRs (Lenz et al, 1984; Speck et
al, 1990) initiate and promote the transcription of host genes located
immediately downstream of the LTRs and may thus serve relevant cellular
25 functions (Stravenhagen and Robins, 1988; Feuchter et al. 1992; Goodchild
et al, 1992; Ting et al, 1992; Schulte et al, 1996).

The human γ -like globin genes consist of the embryonic ϵ the fetal
 γ and δ , and the adult β and δ genes located on Chromosome 11 in a
transcriptional order of 5' ϵ - γ - δ - β - δ -3' (Efstratiadis et al. 1980). The
30 transcription of these genes is regulated by the far upstream Locus Control
Region (LCR), which is defined by four erythroid specific DNase I
hypersensitive sites HS 1, 2, 3 and 4 (Tuan et al, 1985; Forrester et al, 1987;
Grosveld et al, 1987; Dhar et al, 1990). The LCR between HS1 and HS4 is

present in other mammals from mouse to galago and comprises the major functional component of the LCR (reviewed by Hardison et al, 1997). A ubiquitous HS5 site has been identified further upstream of the HS 1-4 sites (Tuan et al, 1985; Dhar et al, 1990) in the apparent 5' boundary area of the LCR.

Enhancer elements are cis-acting and increase the level of transcription of an adjacent gene from its promoter in a fashion that is relatively independent of the position and orientation of the enhancer element. In fact, Khoury and Gruss, 1983, Cell 33:313, state that "the remarkable ability of enhancer sequences to function upstream from, within, or downstream from eukaryotic genes distinguishes them from classical promoter elements . . . " and suggest that certain experimental results indicate that "enhancers can act over considerable distances (perhaps >10 kb)."

Enhancer elements have been identified in a number of viruses, including polyoma virus, papilloma virus, adenovirus, retrovirus, hepatitis virus, cytomegalovirus, herpes virus, papovaviruses, such as simian virus 40 (SV40) and BK, and in many non-viral genes, such as within mouse immunoglobulin gene introns. Enhancer elements may also be present in a wide variety of other organisms. Host cells often react differently to different enhancer elements. This cellular specificity indicates that host gene products interact with the enhancer element during gene expression.

Although gene replacement by homologous recombination could be used instead of integrating vectors, this approach is not yet technically practical because of the very low success rate of the homologous recombination events and the inability to culture the pluripotent stem cells required for this approach.

BRIEF SUMMARY OF THE INVENTION

Disclosed are an enhancer, insulator, and promoter from the HS5 region in the 5' boundary area of the locus control region of human γ -like globin genes. These transcription control sequences can be used to control expression of any desired gene of interest and can be used in any vector for this purpose. The control sequences are derived from the area in and around

the U3 region of a solitary endogenous retrovirus (ERV) 9 long terminal repeat (LTR).

Also disclosed are methods of expressing any gene of interest. For this purpose, the control sequences can be operably linked to the gene of interest (and operably linked to each other). The disclosed enhancers, insulators, and promoters can also be used with any other control sequences. Preferably, the control sequences are used in vectors to obtain expression of a gene of interest in a cell, including cells in animals.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the location and structure of the ERV LTR in the boundary area of the β -globin LCR. The top line shows the human β -like globin gene locus. Solid Boxes are the embryonic ϵ -, fetal γ - and adult δ - and β -globin genes. The vertical arrows indicate locations of the DNase I hypersensitive sites HS 1, 2, 3, 4 and 5. The hatched box 5' of the HS5 site is a solo ERV-9 LTR. The hatched box 3' of the β -globin gene is a second copy of the ERV-9 LTR located 30 kb 3' of the β -globin gene (Henthorn et al, 1986; Anagnou et al, 1995). The middle line is the enlarged 5' boundary area drawn to scale according to the 1 kb scale bar. Open, hatched and gray boxes are respective locations of the HS5 site, ERV-9 LTR and an arbitrary upstream region (Ups) which was used as a control sequence for the LTR in reporter gene assays and RT-PCR studies. The bottom line is the structure of the LTR. Short horizontal arrows are the 14 short tandem repeats in the U3 region. Solid bar is the R region. Long horizontal arrows are the three longer repeats in the U5 region.

Figures 2A and 2B is the sequence of the 5'HS5 LTR in the 5' ϵ 1.4 phage DNA clone from K562 cells (SEQ ID NO:1). The four bases GTAT with the heavy overline and underline located at the 5' and 3' ends of the LTR are the presumed integration site of the LTR in the human genomic DNA. The horizontal arrows in U3 are the 14 tandem repeats of 37-41 bases in the U3 region. Angled arrow is the presumed transcriptional initiation site in the LTR, marking the beginning of the R region. The long horizontal arrows in the U5 region are the three repeats of 70 bases in U5. Arrowheads

connected to dotted overlines are locations of the PCR primers used in DNA PCR and RT-PCR studies discussed in Example 1. Directions of the arrowheads are the 5' to 3' direction of the primers.

Figure 3 is a comparison of the sequences of the U3 repeats. The top line is the organization of the four subtype U3 repeats 1, 2, 3 and 4 in 5'HS5 LTR. P is the promoter in the U3 region. In the middle are the sequences of the subtype repeats 1, 2, 3, and 4 (SEQ ID NOs:8, 9, 10, and 11, respectively). Underlined bases are the GATA, CCAAT, CACCC or CCACC motifs. At the bottom are consensus sequences of the U3 repeats in different ERV-9 LTRs. 5'HS5 (SEQ ID NO:12), 3'β (SEQ ID NO:13) and LTR2 (SEQ ID NO:14) are the 5'HS5 LTR, the LTR at 25 kb 3' of the β-globin gene (Henthorn et al. 1986; Anagnou et al, 1995), and the LTR in a random human DNA clone (Lania et al, 1992), respectively. Lower case letters separated by slashes indicate polymorphic bases in the U3 repeats.

Figure 4 is a sequence comparison of three U3 promoters and the ε-globin promoter. At the top is the U3 promoter of the 5'HS5 LTR (nucleotides 1194 to 1287 of SEQ ID NO:1). The overlined bases are the equivalent of the TATA box (Strazzullo et al, 1994). Underlined bases are the DNA motifs found also in the U3 repeats. Angled arrow is the transcriptional initiation site in LTR2 (La Mantia et al. 1992; Strazzullo et al, 1994) and the presumed transcriptional initiation site in the 5'HS5 LTR. At the bottom is the sequence alignment of the four promoters in the 5'HS5 LTR (nucleotides 1194 to 1287 of SEQ ID NO:1), 3'β LTR (SEQ ID NO:2) and LTR2 (SEQ ID NO:3), respectively. Dashes are DNA base deletions.

Figures 5A-5D is a sequence alignment of the normal human (Hu N: nucleotides 624 to 1781 of SEQ ID NO:1), truncated human (Hu S: SEQ ID NO:6) and gorilla (Gori; SEQ ID NO:7) LTRs. Majority bases represents the consensus DNA sequence among the three LTRs (SEQ ID NO:5). Numbers between two horizontal lines are the DNA base ruler with base 1 being the first base of the first U3 repeat in the LTRs. Vertical arrows are the positions of the first base in the U3 repeats. Dots represent the same bases in the human or gorilla DNAs as those in the consensus sequence. Dashes represent base deletions. The GTAT bases at positions 1081-84

marked with heavy overline are the integration site of the 5'HS5 LTR in both human and gorilla DNAs.

Figure 6 is a diagram comparing the structures of the 5'HS5 LTR in the genomes of human and gorilla and in people of different racial lineages.

5 Hu N is the human LTR of the normal length with 14 U3 repeats. Hu S is the human LTR of a shorter length with 11 U3 repeats. Gori is the gorilla LTR with 5 U3 repeats. Numbers in parentheses are the total number of bases in the LTRs including 140 bases of genomic DNAs downstream of the LTR insertion site—the GTAT bases, that were amplified by the PCR
10 primers. Bent lines in Hu S and Gori are deletions of three and nine complete U3 repeats in the truncated human and gorilla LTRs respectively.

Figure 7 is a diagram of the structure of recombinant CAT constructs. LTR is a 1 kb LTR sequence. Ups is 1.2 kb of DNA upstream of the LTR (see Figure 1). ϵ p is a 200 bp ϵ -globin promoter. HS2 is a 0.74 kb HS2
15 enhancer. HS5 is a 1.2 kb sequence spanning the HS5 site.

Figure 8 is a graph of enhancer and promoter activities (in percent of substrate converted) of the 5'HS5 LTR in recombinant CAT constructs Ups-CAT, HS2- ϵ p-CAT and LTR-CAT plasmids transiently transfected into K562, MEL and HL60 cells. Percent Conv is percentage conversion of the
20 14 C-chloramphenicol substrate by the CAT enzyme produced by the transfected test plasmid after normalization with respect to a common level of a co-transfected CMV- β -gal plasmid.

Figure 9 is a graph of enhancer and promoter activities (in percent of substrate converted) of the 5'HS5 LTR in recombinant CAT plasmids ϵ p-CAT, HS2- ϵ p-CAT, LTR-CAT, LTR- ϵ p-CAT, HS5- ϵ p-CAT and LTR-HS5-
25 ϵ p-CAT integrated into the genome of K562 cells. Percent Conv is the percentage conversion of the 14 C-chloramphenicol substrate by the CAT enzyme produced by the integrated plasmids after normalization with respect to the per cell copy numbers of the plasmids.

30 Figure 10 is a diagram of the 5'HS5 LTR in normal human DNA with 14 U3 enhancer repeats. The four horizontal lines 1, 2, 3 and 4 represent the anticipated RT-PCR fragments amplified respectively by Primer pairs 1-4,

synthesized according to the K562 sequence in Figure 2. Numbers below the lines are the anticipated sizes in base pairs of the amplified cDNA fragments.

Figure 11 is a diagram of examples of constructs using the disclosed enhancers and promoters.

5 Figure 12 is a diagram of examples of constructs using the disclosed enhancers and promoters.

Figure 13 is a diagram of examples of constructs using the disclosed insulators.

DETAILED DESCRIPTION OF THE INVENTION

10 Transcription of the human β -like globin genes in erythroid cells is regulated by the far-upstream locus control region (LCR). Five kilobases of new upstream DNA were cloned and sequenced in order to define the 5' border of the LCR. An LTR-retrotransposon belonging to the ERV-9 family of human endogenous retroviruses was found in the apparent 5' boundary
15 area of the LCR. This ERV-9 LTR contains an unusual U3 enhancer region comprised of fourteen tandem repeats with recurrent GATA, CACCC and CCAAT motifs. This LTR is conserved in human and gorilla, indicating its evolutionary stability in the genomes of primates. In both recombinant constructs and the endogenous human genome, the LTR enhancer and
20 promoter activate the transcription of cis-linked DNA preferentially in erythroid cells.

Sequencing data of the 5' border region of the LCR reveal a solitary ERV-9 LTR with the characteristics of a retrotransposon in a location near the HS5 site (see Figure 1). This 5' HS5 LTR possesses an unusual
25 sequence feature in the U3 enhancer region which is comprised of fourteen tandem repeats of a consensus DNA of 41 bases. These U3 repeats as well as the downstream promoter contain recurrent GATA, CACCC and CCAAT motifs. This LTR-retrotransposon is conserved with 98-99% sequence identities in people of different races and in the gorilla, except that some
30 people have eleven instead of fourteen U3 repeats and the gorilla has only five U3 repeats. Functional tests with the CAT reporter gene assays demonstrate that the human 5' HS5 LTR activates the cis-linked CAT gene and possesses enhancer and promoter activities in erythroid cells. In the

CAT reporter gene assays, the LTR also synergized with and activated the cis-linked HS5 site. Consistent with these results, RT-PCR studies of cellular RNAs isolated from human primary cells and cell lines indicate that the endogenous LTR activates transcription of the downstream R, U5 and the genomic DNA at a higher level in erythroid than in nonerythroid cells.

Disclosed are enhancers, insulators, and promoters derived from the HS5 region in the 5' boundary area of the locus control region of β -like globin genes. These transcription control sequences can be used to control expression of any desired gene of interest and can be used in any vector for this purpose. The control sequences are derived from the area in and around the U3 region of a solitary endogenous retrovirus long terminal repeat (ERV-9 LTR).

Also disclosed are methods of expressing any gene of interest. For this purpose, the control sequences can be operably linked to the gene of interest (and operably linked to each other). The disclosed enhancers, insulators, and promoters can also be used with any other control sequences. Preferably, the control sequences are used in vectors to obtain expression of a gene of interest in a cell, including cells in animals.

Current strategies for gene expression in mammals and mammalian cells, especially gene therapy of hereditary or acquired blood diseases, employ retrovirus-mediated gene-transfer techniques. One of the common problems of this approach has been the extinction of the expression of the transgenes by the long terminal repeats (LTRs) of the vector flanking the therapeutic transgene and by the host sequences flanking the LTR-transgenic cassette. The disclosed enhancers--derived from the powerful enhancer discovered in the solitary LTR of the ERV-9 human endogenous retrovirus located in the 5' border of the β -globin Locus Control Region--can alleviate this problem. The ERV-9 LTR-enhancer is most active in erythroid cells and can thus be used to replace the LTR in the retroviral vector to avoid the transcriptional silencing of the transgene and to boost the transcription of the therapeutic transgene in erythroid progenitor cells. Another problem with gene expression in animal and mammalian cells, interference from flanking transcription, can be alleviated using the disclosed insulator. The disclosed

insulators are derived from a stretch of LTR DNA of 600 bases, which contains a very high G and C bases of 70% and is located immediately upstream of the ERV-9 LTR enhancer. The disclosed insulators can be used to insulate expression cassettes, especially those to be inserted in the genome of the host cell, from the transcriptional interference and silencing of the flanking host sequences.

The solitary ERV-9 LTR sequence in the β -globin Locus Control Region belongs to middle repetitive sequences in the human genome with a haploid copy number of 3000-4000. The first copy of a solitary ERV-9 LTR was reported in 1989. The functional significance of the ERV-9 LTRs dispersed in the human genome may be to transcriptionally activate and thus mark the cis-linked loci of hematopoietic genes and gene families in early progenitor cells during ontogeny and hematopoietic lineage differentiation and the specific function of the solo ERV-9 LTR located near the HS5 site in the 5' border of the human β -globin locus control region (LCR) may initiate transcription of the LCR during early stages of ontogeny and this transcription process of the LCR regulates the transcriptional activation of the further downstream β -like globin genes during erythropoiesis.

Specifically disclosed are nucleic acid molecules comprising all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3 enhancer, where a functional portion is a portion of the U3 enhancer that retains enhancer function. Also disclosed are nucleic acid molecule comprising all or a functional portion of the U3 insulator (nucleotides 5 to 594 of Figure 2; nucleotides 5 to 594 of SEQ ID NO:1), or modified forms of the U3 insulator, where a functional portion of the U3 insulator is a portion of the U3 insulator that retains insulator function. Also disclosed are nucleic acid molecules comprising (1) all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3 enhancer, operably linked to (2) all or a functional portion of the U3 insulator (nucleotides 5 to 594 of Figure 2; nucleotides 5 to 594 of SEQ ID NO:1), or modified forms of the U3 insulator, where a functional portion is a portion of the U3 enhancer that

retains enhancer function and where a functional portion of the U3 insulator is a portion of the U3 insulator that retains insulator function.

Also disclosed are nucleic acid molecules comprising all or a functional portion of the U3 promoter (nucleotides 1194 to 1322 of Figure 2; 5 nucleotides 1194 to 1322 of SEQ ID NO:1), or modified forms of the U3 promoter, where a functional portion of the U3 promoter is a portion of the U3 promoter that retains promoter function. Also disclosed are nucleic acid molecules comprising (1) all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID 10 NO:1), or modified forms of the U3 enhancer, operably linked to (2) all or a functional portion of the U3 promoter (nucleotides 1194 to 1322 of Figure 2; nucleotides 1194 to 1322 of SEQ ID NO:1), or modified forms of the U3 promoter, where a functional portion is a portion of the U3 enhancer that retains enhancer function and where a functional portion of the U3 promoter 15 is a portion of the U3 promoter that retains promoter function.

Also disclosed are nucleic acid molecules comprising the U3 R region (nucleotides 1322 to 1380 of Figure 2; nucleotides 1322 to 1380 of 20 SEQ ID NO:1), or modified forms of the U3 R region. Also disclosed are nucleic acid molecules comprising (1) all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3 enhancer, operably linked to (2) the U3 R region (nucleotides 1322 to 1380 of Figure 2; nucleotides 1322 to 1380 of SEQ ID NO:1), or modified forms of the U3 R region, where a functional portion is a portion of the U3 enhancer that retains enhancer 25 function.

Also disclosed are nucleic acid molecules comprising (1) all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3 enhancer; operably linked to (2) all or a functional portion of the U3 30 insulator (nucleotides 5 to 594 of Figure 2; nucleotides 5 to 594 of SEQ ID NO:1), or modified forms of the U3 insulator; and operably linked to (3) all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3

enhancer; where a functional portion is a portion of the U3 enhancer that retains enhancer function, where a functional portion of the U3 insulator is a portion of the U3 insulator that retains insulator function, and where a functional portion of the U3 promoter is a portion of the U3 promoter that

5 retains promoter function.

Enhancers

The disclosed enhancers have enhancer function. Enhancers function to increase the transcription from promoters in proximity to the enhancer. The disclosed enhancers, like many enhancers, can function both upstream

10 and downstream from a gene, and in either orientation. The disclosed enhancers are, or are derived from, all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of Figure 2; nucleotides 595 to 1193 of SEQ ID NO:1), or modified forms of the U3 enhancer, where a functional portion is a portion of the U3 enhancer that retains enhancer function. The

15 disclosed enhancers can be combined with other transcription control elements, including the disclosed insulators and promoters.

Disclosed are primate 5' HS5 ERV-9 LTR enhancers. In particular, human and gorilla 5' HS5 ERV-9 LTR enhancers are disclosed. A preferred form of enhancer is the U3 enhancer present on nucleotides 595 to 1193 of

20 Figure 2 (nucleotides 595 to 1193 of SEQ ID NO:1). The U3 enhancer is made up of fourteen repeat units, where each repeat has one of the following four sequences:

TATCTAGCTCAGGGATTGTAAATACACCAATCGGCAGTCTG (SEQ ID NO:8),

25 TGTCTAGCTCAAGGTTTGTAAACACACCAATCAGCACCC1G (SEQ ID NO:9),

TATCTAGCTCAGGGTTTGTGAATGCACCAATCAACACTCTG (SEQ ID NO:10),

TGTCTAGCTACTCTGTGGGGACGTGGAGAACCTTTA (SEQ ID

30 NO:11).

Also disclosed are modified forms of the U3 enhancer where the modified enhancer retains enhancer function.. These include:

Enhancers having three or more repeats, where each repeat has one of the following sequences:

- TRTCTAGCTCADGGTTTGTRAAYRCACCAATCAGCACTCTG (SEQ ID NO:12),
- 5 TATCTAGCTCAGGGATTGTAAATACACCAATCGGCAGTCTG (SEQ ID NO:8),
- TGTCTAGCTCAAGGTTTGTAACACACCAATCAGCACCTG (SEQ ID NO:9),
- TATCTAGCTCAGGGTTTGTAATGCACCAATCAACACTCTG (SEQ ID NO:10),
- 10 TGTCTAGCTACTCTGTGGGGACGTGGAGAACCTTTA (SEQ ID NO:11).

Enhancers having three or more repeats, where each repeat has one of the following sequences:

- 15 TATCTAGCTCAGGGATTGTAAATACACCAATCGGCAGTCTG (SEQ ID NO:8),
- TGTCTAGCTCAAGGTTTGTAACACACCAATCAGCACCTG (SEQ ID NO:9),
- TATCTAGCTCAGGGTTTGTAATGCACCAATCAACACTCTG (SEQ ID NO:10),
- 20 TGTCTAGCTACTCTGTGGGGACGTGGAGAACCTTTA (SEQ ID NO:11).

Enhancers having three or more repeats, where each repeat has the following sequence:

- 25 TRTCTAGCTCADGGTTTGTRAAYRCACCAATCAGCACTCTG (SEQ ID NO:12).

Enhancers where the enhancer has from three to fourteen repeat units.

- Enhancers where one or more of the repeat units of the enhancer are deleted, one or more of the repeat units are replaced with a repeat unit of the enhancer having a different sequence than the repeat unit that is replaced,
- 30 one or more repeat units of the enhancer are added to the enhancer, or a combination of one or more of these modifications.

The disclosed control sequences can be used, alone or in combination, to express any gene of interest. For this purpose, the control sequences can be operably linked to the gene of interest. Preferably, the gene encodes a protein. Preferably, the control sequences are used in vectors to obtain expression of a gene of interest in a cell, including cells in animals. Preferred vectors include retroviral vectors, adenoviral vectors, and other vectors suitable for gene expression in mammalian cells and/or suitable for gene therapy. Many vectors are known and the disclosed control sequences can be used in any of these vectors.

Also disclosed are cells transformed with vectors containing one or more of the disclosed control sequences. That is vectors containing one or more of the disclosed enhancers, insulators, or promoters. Preferred cells are eukaryotic cells, animal cells, and mammalian cells. Also disclosed is a method of expressing a protein, the method comprising culturing cells transformed with a vector containing one or more of the disclosed control sequences operably linked to the gene. Also disclosed is a method of expressing a gene in an animal, the method comprising introducing into the animal cells transformed with a vector containing one or more of the disclosed control sequences operably linked to the gene. Also disclosed is a method of expressing a gene in an animal, the method comprising introducing into cells of an animal a vector containing one or more of the disclosed control sequences operably linked to the gene.

Insulators

Insulators are nucleic acid segments that reduce or eliminate transcription from adjacent regions from affecting the nucleic acid segment to which the insulator is associated. The disclosed insulators preferably are placed upstream of other control sequences and/or downstream of genes. Insulators are preferably placed between different genes, transcription units, or genetic domains to reduce or prevent interference of the adjacent expression sequences. The disclosed insulators are, or are derived from, all or a functional portion of the U3 insulator (nucleotides 5 to 594 of Figure 2; nucleotides 5 to 594 of SEQ ID NO:1), or modified forms of the U3

insulator, where a functional portion of the U3 insulator is a portion of the U3 insulator that retains insulator function.

Promoters

Promoters are nucleic acid segments that mediate initiation of transcription. The disclosed promoters are, or are derived from, all or a functional portion of the U3 promoter (nucleotides 1194 to 1322 of Figure 2; nucleotides 1194 to 1322 of SEQ ID NO:1), or modified forms of the U3 promoter, where a functional portion of the U3 promoter is a portion of the U3 promoter that retains promoter function.

Use Of Control Elements

The disclosed enhancers, insulators, and promoters can be used in a variety of vectors and expression constructs to regulate and promote transcription of genetic elements placed in the same constructs. The disclosed control elements are preferably used in retroviral vectors to obtain expression in mammalian cells, and especially to express genes in cells in, or to be introduced into, animals (including humans) for gene therapy.

Specific examples of such uses are:

1. The 5'HS5 ERV-9 LTR and/or its component U3 enhancer, insulator, and promoter, the R and the U5 regions can be used to replace the LTRs or their equivalent U3, R and U5 regions of retroviral vectors designed for gene therapy of hereditary or acquired hematological diseases including sickle cell disease, thalassemias, leukemias and AIDS.
2. The U3 enhancer, insulator, and promoter, and the R region can be used to activate (and/or insulate) in hematopoietic cells the transcription of a cis-linked transgene in either viral or non-viral vectors. The host cells for the transgene can be the hematopoietic stem cells, progenitor cells or mature lineage differentiated cells such as the erythroid, myeloid or lymphoid cells.
3. Base mutations, and/or rearrangements and substitution of repeat units, can be introduced into the U3 and R regions to enable the U3 enhancer and promoter and the R region to work more efficiently in a specific hematopoietic lineage such as the erythroid, myeloid or lymphoid lineage.

Design of the retroviral vectors and transgenic cassettes.

1. The disclosed enhancers, promoters, R region, and U5 region can be used to replace the LTRs or their component U3, R and U5 regions of retroviral vectors designed for gene therapy of hereditary or acquired hematological diseases. The disclosed insulators can also be added to the vector. The replacement can be in either the 5' or the 3' LTR or both the 5' and 3' LTRs of an appropriate retroviral vector. Example constructs are shown in Figure 11.

U3: the U3 enhancer and promoter of the 5'HS5 ERV-9 LTR

10 **R:** the R region of the 5'HS5 ERV-9 LTR

U5: the U5 region of the 5'HS5 ERV-9 LTR

U3E: the U3 enhancer of the 5'HS5 ERV-9 LTR

U3p, R and U5: the U3 promoter, R and U5 regions of appropriate non-5'HS5 ERV-9 LTRs.

- 15 2. Constructs such as those shown in Figure 12 can be used to activate the transcription of cis-linked transgene spliced in either viral or non-viral vectors in hematopoietic cells.

U3: the U3 enhancer and promoter of the 5'HS5 ERV-9 LTR

R: the R region of the 5'HS5 ERV-9 LTR

20 **U5:** the U5 region of the 5'HS5 ERV-9 LTR

U3E: the U3 enhancer of the 5'HS5 ERV-9 LTR

U3P: the U3 promoter of the 5'HS5 ERV-9 LTR

R and U5: the R and U5 regions of appropriate non-5'HS5 ERV-9 LTRs.

P: appropriate promoter other than the U3 promoter of the 5'HS5 ERV-9 LTR.

- 25 3. The disclosed insulators can be used to insulate integrated transgenes in hematopoietic and non-hematopoietic cells from transcriptional interference exerted by the host genome and or elimination by the host genome over time, so that the transgene can be efficiently transcribed from its own enhancer and promoter and also can be stably integrated in the host genome over time. Examples of constructs using the disclosed insulators are shown in Figure 13. Such constructs will have improved expression

consistency and stability by limiting or eliminating the influence of flanking transcription activities.

The U3 enhancer repeats of the 5'HS5 LTR can also be used to identify transcription factors that bind to the enhancer. The transcription factors bound by the DNA motifs in U3 repeats can be identified by electrophoretic mobility shift assays (EMSA) with nuclear extracts isolated from cells, such as K562 and placenta trophoblasts, and supershift assays with antibodies against various known transcription factors. Such techniques for use with other protein binding sites are well established and can be used with the disclosed enhancers.

The genes encoding new transcription factors identified through this process can then be cloned. The molecular architecture and activity of the U3 enhancer complex can also be examined by site-directed mutagenesis of the U3 repeats in test plasmids containing the Green Fluorescent Protein (GFP) reporter gene, following transfection into cells, such as K562, CFU-E and placental trophoblast cells.

Constructs and Vectors

The disclosed control elements (that is, the disclosed enhancers, insulators, and promoters) are useful for expression of any desired gene. For this purpose, the disclosed control elements can be included in constructs and vectors designed for expression of genes of interest. Many such vectors are known. Preferred vectors are those for use in animal cells, and in particular, those for use in mammalian cells.

Examples of vectors and delivery techniques that can be adapted for use with the disclosed control elements are described in U.S. Patent No. 5,968,735, U.S. Patent No. 5,965,440, U.S. Patent No. 5,965,358, U.S. Patent No. 5,932,210, U.S. Patent No. 5,925,565, U.S. Patent No. 5,888,820, U.S. Patent No. 5,888,767, U.S. Patent No. 5,886,166, U.S. Patent No. 5,871,997, U.S. Patent No. 5,866,696, U.S. Patent No. 5,866,411, U.S. Patent No. 5,858,744, U.S. Patent No. 5,856,152, U.S. Patent No. 5,837,503, U.S. Patent No. 5,830,727, U.S. Patent No. 5,817,492, U.S. Patent No. 5,814,482, U.S. Patent No. 5,811,260, U.S. Patent No. 5,795,577, U.S. Patent No. 5,789,244, U.S. Patent No. 5,783,442, U.S. Patent No. 5,770,400,

U.S. Patent No. 5,759,852, U.S. Patent No. 5,756,264, U.S. Patent No. 5,753,499, U.S. Patent No. 5,744,133, and U.S. Patent No. 5,710,037.

Gene Therapy

The disclosed control elements can be used in vectors and constructs for gene therapy. "Gene therapy" refers to the treatment of pathologic conditions by the addition of exogenous nucleic acids to appropriate cells within the organism. The disclosed control elements can be used to express and increase the efficiency of expression of genes added in gene therapy. Nucleic acids must be added to the cell, transfected or transfected, such that they remain functional within the cell. The disclosed insulators can protect introduced genes from interfering endogenous transcription at the site of insertion. For most gene therapy strategies, the new nucleic acids are designed to function as new genes, i.e., code for new RNA or messenger RNA, which in turn codes for new protein. Alternatively, therapeutic genes can produce antisense or ribozymes which can directly effect cellular or pathogen functions without having to express protein from mRNA. Gene therapy can be directed towards monogenetic disorders like adenosine deaminase deficiency and cystic fibrosis or to polygenetic somatic disorders like cancer.

Human gene therapy has been successfully applied to correct genetic diseases in adenosine deaminase deficiency (severe combined immunodeficiency) (Approved Protocol) "Treatment of Severe Combined Immunodeficiency Disease (SCID) Due to Adenosine Deaminase (ADA) Deficiency with Autologous Lymphocytes Transduced with a Human ADA Gene" Hum. Gene Ther. 1:327-362 (1990); Anderson, W.F. "Human Gene Therapy" Science 256:808-813) and familial cholesterolaemia (Grossman, et al. Nature Genetics 6:335-341 (1994)). Many new gene therapy protocols are in progress or being planned (Morgan and Anderson Ann. Rev. Biochem. 62:191-217 (1993)). Vectors, constructs, and protocols described in the studies above can be adapted for use with the disclosed control elements.

The rapid implementation of gene therapy in human trials has been made possible by the development of relatively efficient means of transferring new nucleic acids into cells, a process generally referred to as "gene transduction". The clinically applicable gene transduction methods fall into

one of three categories: a) cationic lipids, (b) molecular conjugates and (c) recombinant viruses. These different means of accomplishing gene transfection have been recently reviewed by Morgan, *Ann. Rev. Biochem.* 62:191 (1992); Mulligan *Science* 260:926 (1993); and Tolstoshev *Ann. Rev. Pharm. Toxicol.* 32:573 (1993)). Any of these transfer systems can be used for constructs using the disclosed control elements.

Most of the successful human gene therapy protocols utilize vectors derived from defective murine leukemia retroviruses (Anderson *Science* 256:808-813 (1992); Miller *Nature* 357:455-460 (1992); Miller *Curr. Top. Microbiol. Immunol.* 158:1-24 (1992), for review of these vectors and the packaging cell lines, Miller, *Methods in Enzymology* 217:581-599 (1993)). Although there is a limitation in the size of the gene (up to 7 to 8 kb) that can be transduced, the retrovirus based vectors have the advantage in that they can incorporate a permanent copy of the delivered gene into the chromosomes of the recipient cells and therefore potentially can represent a cure for a disorder arising due to the expression of an undesirable protein, activation of an oncogene, or insufficient expression or expression of a defective protein. Due to their retroviral origins, the disclosed control elements are particularly suited for use in retroviral vectors.

The majority of the gene transfer procedures used to date for human gene therapy is known as an ex vivo gene transfer. The recipient cells are removed from the patient and grown in a cell culture laboratory. Replication-incompetent, virus-like particles containing the therapeutic gene, which are produced from packaging cells, are used to transduce the recipient cells. The transduced recipient cells are then selected by growing in selection media, expanded and returned to the patient. The packaging cells are genetically engineered cell lines that, once a therapeutic gene is transferred into the cells, produce virus-like particles containing the therapeutic gene to be delivered into other cells.

Other gene transferring vehicles in which the disclosed control elements can be used are those based on human immunodeficiency virus (HIV) (Poznansky, et al. *J. Virol.* 65:532-536 (1991); Buchschacher, et al. *J. Virol.* 66:2731-2739 (1992); Shimada, et al. *J. Clin. Invest.* 88:1043-1047

(1991)) and adeno-associated virus (Chatterjee, et al. Science 258:1485-1488 (1992); Muzyczka Curr. Top. Microbiol. Immunol. 158:97-129 (1992)).

An HIV based delivery system is believed to be particularly suitable for gene therapy against AIDS. Not only can the genes transferred by HIV virus-based vectors be integrated into the genome of non-dividing cells (Weinberg, et al. J. Exp. Med. 174:1477-1482 (1991); Bukrinsky, et al. Proc. Natl. Acad. Sci. U.S.A. 89:6580-6584 (1992); Lewis, et al. [published erratum appears in EMBO J. Nov:11(11):4249 (1992)] EMBO. J. 11:3053-3058 (1992)), the presence of HIV gp120 on the surface of the gene delivering particles renders them specific for gene delivery to CD4⁺ cells.

The U3 enhancer region in 5' HS5 LTR contains an unusual sequence of fourteen tandem repeats of 37-41 bases. The tandem repeats are comprised of four subtypes 1, 2, 3 and 4, which are arranged in the LTR in the order 1-2-3-4-1-2-3-4-1-2-3-4-4-1. The consensus sequence of the U3 repeats (SEQ ID NO:12) reveals five conserved motifs, GATA, TAGCTCA, GGTTTGT (or GGTGG/CCACC in subtype 4) and CCAAT. The motifs GATA, CCAAT and CACC can potentially bind to cognate transcription factors abundantly expressed in hematopoietic and erythroid cells.

The consensus sequence of U3 repeats shows higher than 90% sequence homology with that of the U3 repeats of the 3' ERV-9 LTR located 25 kb 3' of the γ -globin gene and of LTR2, a random clone of ERV-9 LTR (Figure 3).

The promoter sequence in the LTR is located in the U3 region at the 3' end of the U3 repeats and is immediately upstream of the transcribed R region whose 5' border marks the transcriptional initiation site for retroviral RNA synthesis. The promoter of the 5'HS5 LTR shows a sequence homology of 80% with the promoter of the 3' LTR and of over 90% with the promoter of LTR2. The transcriptional initiation site of LTR2 has been determined by primer extension to be located 28 bases downstream of the AATAAAA box. Because of extensive sequence homologies between the 5'HS5 LTR and the LTR2 promoters, especially the 100% sequence homology in the 70 DNA bases flanking the AATAAAA box, the transcriptional initiation site of the 5'HS5 LTR was placed at the identical T

base 28 bases downstream of the AATAAAA box. All three LTR promoters contain the GATA, CACCC and CCAAT motifs at identical locations, -36, -46 and -63 bases respectively, relative to the retroviral transcriptional initiation site.

5 The 5'HS5 LTR promoter also bears structural similarities with the promoters of the further downstream α -, β - and γ -globin genes in that a combination of similar GATA, CACCC and CCAAT motifs is found also upstream of the AATAAAA boxes in the globin promoters. In particular, the 5'HS5 LTR and the γ -globin promoter share additional sequence homologies
10 in the region immediately 5' of the transcriptional initiation site. The above homologies indicate that, like the globin promoters, the 5'HS5 LTR enhancer and promoter ought to be active in erythroid cells. Indeed, transfection assays show that the 5'HS5 LTR exhibits enhancer and promoter activities and can promote the transcription of cis-linked DNA to relatively high levels
15 in erythroid cells and in placenta.

 The consensus sequence of the modular U3 repeats in 5'HS5 LTR reveals that the modular U3 repeat contains five well conserved and recurrent DNA motifs organized invariably in the following 5'→3' order: GATA, TAGCTCA, GGTTTGT (or TGGTGGG in subtype 4) and
20 CACCAATCAGCA (nucleotides 25 to 36 of SEQ ID NO:12). This invariable sequence structure suggests a definitive organization of the cognate protein factors in the assembly of the U3 enhancer complex.

 The GATA motifs bind to the GATA family of transcription factors including GATA-1, -2 and -3. Targeted disruptions of the GATA-1, -2 and -
25 3 genes have been reported to cause severe abnormalities in hematopoiesis and erythropoiesis, indicating that these factors play important regulatory roles in erythroid cells. Different GATA factors are expressed at relatively higher levels in different hematopoietic cells. In CD34+ hematopoietic stem/progenitor cells, GATA-2 is expressed at a high level relative to
30 GATA-3 and GATA-1. In erythroid K562 cells, both GATA-1 and GATA-2 are expressed. In CFU-E, GATA-1 is the major detected GATA factor. In placenta trophoblasts, GATA-2 and GATA-3 are expressed.

The CACCC motifs bind to erythroid transcription factors EKLF and BKLF. EKLF is expressed at very low levels in K562 cells expressing the embryonic globin program and at much higher levels in MEL cells expressing the adult globin program. Unlike EKLF, BKLF is expressed abundantly in embryonic yolk sac and fetal liver and is not confined to erythroid cells. However, the motif in the U3 repeats is CACC and not CACCC found in the strong EKLF and BKLF binding sites, and may thus bind to these factors weakly or bind to different factor(s).

The CCAAT motifs may bind to two families of protein factors, the C/EBPs expressed in various hematopoietic cells and adipocytes and the ubiquitous NF-Y complex. The C/EBP transcription factors include C/EBP β , δ , ϵ , and CHOP, a dominant negative inhibitor of the C/EBPs. They bind to the CCAAT motifs as a homodimer or heterodimer through the -ZIP domain. The CCAAT boxes have been reported to play pivotal roles in the activities of the globin promoters, suggesting the existence in erythroid cells of transcription factors that bind to and activate the CCAAT boxes. However, none of the C/EBP β , δ , and ϵ are present at detectable levels in erythroid K562 cells and C/EBP γ , a ubiquitous factor, appears to be expressed mainly in lymphoid cells. This suggests that in K562 cells the CCAAT box may be bound paradoxically by negative regulators CHOP and CDP or primarily by the ubiquitous NF-Y complex.

The NF-Y complex, also named CP1, consists of three subunits A, B and C. All three subunits are required for binding to the CCAAT box as a trimeric complex through the histone fold motif, which bears similarity to the DNA binding domain of the histones. The NF-Y factors through the histone fold domain may also associate with histone acetyltransferase and thus be able to remodel and open up the chromatin structure of the CCAAT box and its neighboring DNA. In EMSA gels with nuclear extract from erythroid cells, after the NF-Y complex was supershifted with antibodies, the CCAAT box containing probe still formed shifted complexes. This suggests that erythroid cells may contain yet unidentified nuclear factors that may bind to the CCAAT motifs in U3 repeats.

The remaining two conserved sequence motifs TAGCTCA and GGTTTGT in the U3 repeats may also be bound by yet unidentified transcription factors present in erythroid cells. It is of interest to note that motifs similar to TAGCTCA are found also in enhancers and promoters of genes expressed in various hematopoietic lineages: TAGCCTGA in the MLV U3 enhancer, TAGCTAA in the promoter of M-CSF receptor gene and TAGCTTCA in the Invariant Chain promoter of the major histocompatibility complex.

The enhancers of many genes including the HS2 enhancer of the globin LCR usually span several hundred bases and are bound by many different protein factors, which make the analysis of the enhancer complex a complicated task. In contrast, the 14 modular U3 repeats in the 5'HS5 LTR contain up to four well conserved DNA motifs and may be bound by similarly limited number of recurrent protein factors, making it a simpler task to analyze the structure of this enhancer complex.

Example

This example describes the cloning and characterization of the 5' border region of the LCR upstream of human β -like globin genes.

MATERIALS AND METHODS

Isolation of 5' 1.4 phage clone and DNA sequencing: The 5' 1.4 phage clone spanning 12 kb of DNA 5' of the HS4 site was obtained from a K562 genomic DNA library constructed in EMBL phage (Weber-Benarous et al, 1988). The library was screened with a unique DNA probe 5' 1.4 located near the HS4 site in the LCR (Li et al, 1985). The genomic DNA insert contained 8 kb of DNA spanning the HS5 site whose sequence was subsequently reported (Yu et al, 1994) and 5 kb of further upstream new DNA. The 8 kb of DNA was cleaved by Hind III into four sub-fragments of 2.7 kb spanning the HS5 site and 1.5, 1.6 and 2 kb spanning the new DNA. They were subcloned into a plasmid vector (Tuan et al. 1990) and sequenced with the dideoxy terminator method (Sanger et al, 1977) using Sequenase or Taquenase Kit (USB Corp). This sequence strategy produced unambiguous DNA sequencing ladders for the entire 8 kb of DNA except for the 1 kb of

DNA in the junction area between the 1.5 and 1.6 kb subclones which contained the repetitive sequences of the ERV-9 LTR. The junction DNA was recloned into a phagemid vector Bluescript II SK(+/-) (Stratagene) and the single stranded DNA was sequenced as above. The sequences were assembled and analyzed using the GCG DNA analysis software. The 8 kb DNA sequence was submitted to GenBank (BankIt 193637 AF064190).

Purification of genomic DNAs from the gorilla and people of different races: Genomic DNAs were isolated anonymously from human blood samples collected by the Hemoglobin Laboratory at the Medical College of Georgia for diagnosis of thalassemia and sickle cell disease. African samples were from patients homozygous for sickle cell disease or Hereditary Persistence of Fetal Hemoglobin (HPFH), Arabic and Asian samples were from people hemizygous for α -thalassemia and the Caucasian samples were from normal individuals or patients with β -thalassemia. The gorilla blood sample was obtained from the Yerkes Primate Center of Emory University. High molecular weight genomic DNAs were purified from nucleated blood cells (Poncz et al, 1982)

PCR-amplification of the 5'HS5 LTR in genomic DNAs and sequence analysis of the amplified LTR: The 5'HS5 LTRs were amplified from genomic DNAs with Primer pair 3 used also for RT-PCR (Figure 10; forward primer, positions 595-616 and reverse primer 1807-1831, Figure 2; nucleotides 595 to 616 of SEQ ID NO:1). PCR conditions consist of an initial denaturation at 95°C for 1.5 min, followed by 32 cycles of denaturation at 95°C for 1.5 min, annealing at 59°C for 1 min and extension at 72°C for 2 min and a final extension step at 72°C for 15 min. The amplified LTR fragments were purified by Quantum Plasmid Miniprep Kit (Bio-Rad) and sequenced by the Molecular Biology Core Laboratory of the Medical College of Georgia using the cycle sequencing technique with fluorescent dideoxy terminators.

Construction of recombinant CAT plasmids: LTR-CAT (Construct 1): The 1 kb LTR was amplified from K562 genomic DNA by PCR with forward primer: 5' TACTGTCGACCTGAGT-TTGCTGGGGATG 3' (positions 3250-3271 in the 8 kb GenBank sequence,

BankIt 193637 AF064190 corresponding to positions 595-616 in Figure 2; nucleotides 595 to 616 in SEQ ID NO:1) and reverse primer 5' GATGGATCCTGTGTCCGGAATTGGTGG 3' (positions 4282-4299 in GenBank sequence; positions 1677-1694 in Figure 2; nucleotides 1677 to 1694 in SEQ ID NO:1). A Sal I and a Bam HI cloning site (underlined) were added respectively to the forward and reverse primers. The PCR fragment was cleaved with Sal I and BAM HI enzymes and together with a Bam HI-Hind III adapter was spliced into a promoterless CAT vector derived from ϵ p-CAT (Construct 3) in which the ϵ -globin promoter (ϵ p) was removed with Sal I and Hind III digestions. Ups-CAT (Construct 2) contains a 1 kb PCR fragment amplified from the genomic DNA located 2 kb further upstream of the LTR and was created with the same cloning strategy. The respective forward and reverse primers were 5' ACTGTCGACTTATGTATTCAAGTTTCG 3' (positions 50-66 in GenBank sequence; SEQ ID NO:21) and 5' GATGGATCCAATAGATTTTTGTCATCT 3' (positions 1203-1220 in GenBank sequence; SEQ ID NO:22). ϵ p-CAT (Construct 3) and HS2- ϵ p-CAT (Construct 4) were previously made (Tuan et al, 1989). LTR- ϵ p-CAT (Construct 5) was created with the above 1 kb LTR DNA obtained by PCR which was cleaved at the Sal I and Bam HI cloning sites and spliced into ϵ p-CAT(Construct 3) which was also cleaved at the Sal I and BAM HI sites located 5' of the ϵ p. HS5- ϵ p-CAT (Construct 6) was created with the same cloning strategy as LTR- ϵ p-CAT. (Construct 5). The 1.2 kb HS5 fragment was generated by PCR from forward primer 5' ACTGTCGACAAGCTTCTGACAAATTATTCTT 3' (positions 5431-5455, GenBank sequence; SEQ ID NO:15) and reverse primer 5' GATGGATCCACTGAAAGGGCTCATGCAAC 3'(positions 6657-6676), GenBank sequence; SEQ ID NO:16). LTR-HS5- ϵ p-CAT (Construct 7) was made from LTR- ϵ p-CAT (Construct 5) which was linearized at the Bam HI site 3' of the LTR. The above 1.2 kb HS5 fragment obtained by PCR was cleaved at the 5' end with Hind III (a natural site) and at the 3' end with Bam

HI and together with a Bam HI-Hind III adapter was spliced into the Bam HI site in LTR- ϵ p-CAT.

Transient and stable transfections and CAT assays: Transfection host cells K562, HL60 and MEL cells were cultured and transfected as described (Tuan et al, 1989) with modifications. In transient transfections, 10 μ g of each of the above CAT plasmids were mixed with 5 μ g of a reference CMV β -gal plasmid and transfected into the host cells by electroporation. CAT assays were carried out as described (Tuan et al, 1989) with two modified steps of normalizations. The CAT extracts were normalized first with respect to the total protein in the extract determined with the BCA (Bicinchoninic acid) protein kit (Pierce) and then with respect to the β -galactosidase level of the co-transfected CMV β -gal plasmid to ensure that the CAT assays of different samples were carried out on extracts containing similar levels of β -gal activities, therefore, similar amounts of the transfected tested plasmids. The β -gal enzyme levels were determined with the β -gal Assay Kit (Promega). The CAT enzymatic activities were analyzed by thin layer chromatography and quantified with a PhosphorImager (Molecular Dynamics). The results were presented as percentages of conversion calculated from the 14 C counts in the acetylated chloramphenicol divided by the total input 14 C counts of the chloramphenicol substrate. In stable transfection, pooled cell populations were studied. The CAT activities were normalized with respect to the copy numbers of the integrated plasmids determined by Southern blots.

Isolation of total cellular RNAs and RT-PCR: Total cellular RNAs were purified from freshly harvested, non-transfected human erythroid K562, promyelocytic HL60, embryonic teratocarcinoma N-Tera (obtained from ATCC) and murine erythroleukemia MEL cell lines, adult human peripheral blood CFU-E and T-lymphocytes (Wickrema et al, 1992) and full term human placenta. The RNAs were purified with the Totally RNA Kit (Ambien). For a semi-quantitative comparison of the RT-PCR bands generated by different primer pairs, each RNA was first reversely transcribed into cDNA with random hexamers as primers into a cDNA master stock,

which was then aliquoted into separate tubes for PCR with different primer pairs as described (Kong et al. 1997). The 5'-->3' sequences of the respective forward and reverse primers are marked in Figure 2. Primer pair 1: CTGAGTTTGCTGGGGATGCGAA (positions 595-616; SEQ ID NO:17) and GATTTAGTGACTCATATTGTTTCTGA (positions 1700-1726; SEQ ID NO:18); Primer pair 2: TGCTGCTGCTCACTGTTTGGGTCTA (positions 1349-1373; SEQ ID NO:19) and the reverse primer was the same as that of Primer pair 1. Primer pairs 3 and 4 contain the same forward primers as the respective forward primers of Primer pairs 1 and 2. Primer pairs 3 and 4 contain a common reverse primer: 5'GGGCACTCTGCCTTAGGGAGTAACA 3' (positions 1807-1831; SEQ ID NO:20). The human β -actin primer pair was obtained from Stratagene. Before RT-PCR, the abilities of the primer pairs to produce amplification fragments were confirmed by PCR with genomic DNA templates.

RESULTS

An LTR-retrotransposon of the ERV-9 family of human endogenous retroviruses is located proximal to the HS5 site in the 5' boundary area of the LCR: In order to study the sequence and function of DNA in the boundary area of the LCR, a K562 DNA library was screened (Weber-Benarous et al, 1988) and obtained a clone containing 8 kb of DNA sequence that spans the HS5 site and 5 kb of new further upstream DNA. As the sequence features of the upstream DNA were previously unknown, the 5 kb new DNA as well as the 3 kb DNA spanning the HS5 site was sequenced (GenBank accession number: BankIt 193637 AF064190). The DNA sequence of the 3 kb DNA spanning the HS5 site is in general agreement with the DNA sequence of this region reported earlier (Yu et al, 1994), except for a number of polymorphic base differences. In the new DNA, sequence matches using the GCG and BLAST programs revealed the existence of a solitary LTR at a location within 2 kb 5' of the HS5 site (Long et al, 1995) (Figure 1). Comparison with a few selected homologous sequences in the GenBank data base, including the LTR sequence located 5' of the ZNF80 protein gene (Di Cristofano et al, 1995. GenBank Accession No. X83497), showed that the 5'HS5 LTR spans 1.7 kb of DNA (Figure 2)

and belongs to the ERV-9 family of human endogenous retroviruses (La Mantia et al, 1991; Lania et al, 1992).

Consistent with a common property of the retrotransposons, the 5'HS5 LTR is flanked by 4 bases of direct repeats GTAT in the genomic DNA immediately 5' and 3' of the LTR sequence (Figure 2). This indicates that the 5' HS5 LTR was inserted into the human ancestral genome at the GTAT site sometime during evolution. In line with the general LTR structure of mammalian retroviruses (Temin, 1982), the 5'HS5 LTR contains the U3, R and U5 regions and is bracketed by the dinucleotides TG and CA respectively at the 5' and 3' ends (Figure 2). The U3 region contains the viral enhancer spanning tandemly repeated DNA sequences and the viral promoter (Lenz et al, 1984; Golemis et al, 1990; La Mantia et al, 1991; Anagnou et al, 1995). The R region starts with the viral transcription initiation site (La Mantia et al, 1992) and is followed by the U5 region (Figure 1). In the U3 region, the 600 DNA bases preceding the U3 repeats are comprised of 70% G and C bases. This GC-rich region is found in many of the homologous ERV-9 LTRs in the data base but is not present in the LTR of the ERV-9 provirus (La Mantia et al, 1991). The U3 enhancer repeats and the promoter in the 5'HS5 LTR show 80-90% base identities with other ERV-9 LTRs found in the human genome (Yang et al, 1983; La Mantia et al, 1991; Lania et al, 1992; Di Cristofano et al, 1995).

It is of interest to note that in addition to the 5'HS5 LTR located approximately 25 kb 5' of the ϵ -globin gene, another ERV-9 LTR is located at a position approximately 25 kb 3' to the β -globin gene (Figure 1). The repetitive DNA in the region 3' of the β -globin gene was first reported by Henthorn et al (1986) and subsequently studied by Anagnou et al (1995). Although neither of those groups recognized that the repetitive DNA was part of an endogenous LTR, sequence matches as shown above revealed that the repetitive DNA of this region bears sequence identities of 80-90% with the U3, R and U5 regions of the 5'HS5 LTR. Thus, two copies of the ERV-9 LTRs exist in flanking positions of the β -globin gene cluster.

Sequence analysis of the U3 enhancer region in the 5' HS5 ERV-9 LTR: The U3 enhancer region of the 5' HS5 LTR shows an interesting

sequence structure. It is comprised of fourteen tandem repeats of a consensus DNA sequence of 37-41 bases (Figure 2). Sequence matches show that the tandem repeats are comprised of four subtypes 1, 2, 3 and 4, which are arranged in the LTR in the order 1-2-3-4-1-2-3-4-1-2-3-4-4-1 (Figure 3). Among the four subtypes, the sequence identities are 60-80%, using subtype 2 as the reference. Among the U3 repeats of each subtype, the sequence identities are 80-98% (Figure 3). The consensus sequence of the fourteen U3 repeats (Figure 3) reveals recurrent sequence motifs that can potentially bind to the GATA (Ko and Engel, 1993); Merika and Orkin, 1993), CCAAT (Johnson and McKnight, 1989) and CACCC (Miller and Bieker, 1993; Crossley et al, 1996) transcription factors. Altogether, the U3 enhancer region contains within 600 bases DNA eight GATA, nine CCAAT, three CACCC and four CCACC sites. The consensus sequence of the fourteen U3 repeats shows higher than 90% sequence identity with that of the seven U3 repeats in the 3'β LTR (Henthorn et al, 1986) and of the six U3 repeats in LTR2, a random clone of the ERV-9 LTR (Lania et al; 1992) (Figure 3).

Sequence analysis of the U3 promoter region: The promoter sequence in the LTR is located in the U3 region at the 3' end of the fourteen U3 repeats. It is located immediately upstream of the R region whose 5' border marks the transcriptional initiation site for retroviral RNA synthesis (Temin, 1982) (Figure 3). The promoter of the 5'HS5 LTR shows a sequence homology of 80% with the promoter of the 3'β LTR and of over 90% with the promoter of LTR2 (Figure 4). The transcriptional initiation site of LTR2 has been determined by primer extension to be located 28 bases downstream of the AATAAAA box (La Mantia et al, 1992; Strazzullo et al, 1994). Because of extensive sequence identities between the 5'HS5 LTR and the LTR2 promoters, especially the 100% sequence homology in the 70 DNA bases flanking the AATAAAA box, the presumptive transcriptional initiation site of the 5'HS5 LTR was placed at the identical T base 28 bases downstream of the AATAAAA box (Figure 4). All three LTR promoters contain the GATA, CACCC and CCAAT motifs located at identical

locations, -36, -46 and -63 bases respectively, relative to the retroviral transcriptional initiation site (Figure 4).

The 5'HS5 LTR promoter also bears structural similarities with the promoters of the further downstream ϵ -, γ - and β -globin genes (Baralle et al, 1980; Shen et al, 1981; Poncz et al, 1983; Li et al, 1985) in that a combination of similar GATA, CACCC and CCAAT motifs is found also upstream of the AATAAAA boxes in the globin promoters (Nienhuis et al, 1984). In particular, the LTR promoter and the ϵ -globin promoter share additional sequence identities in the region immediately 5' of the transcriptional initiation site (Figure 4). The above sequence and structural homologies suggest that, like the globin promoters, the 5'HS5 LTR promoter would be active in erythroid cells.

The 5' HS5 ERV-9 LTR is conserved in the genomes of the gorilla and of people of different racial lineages: As the 5'HS5 LTR is apparently a retrotransposon and is located not near but far upstream of the β -like globin genes, it was possible that the 5'HS5 LTR might have resulted from a recent insertional event in the K562 genome during cell culture and did not serve a relevant cellular function. However, were this the case, the 5'HS5 LTR would not be present in the genome of the gorilla which diverged from the human genome approximately 10 million years ago (Sibley and Ahlquist, 1987) nor in the genomes of people of different racial lineages which diverged approximately 100,000 years ago (Vogel and Motulsky, 1986). To examine this issue, PCR was used to detect the presence or absence of the 5'HS5 LTR in the genomic DNAs isolated from the blood samples of the gorilla and people of different races. The PCR primers were synthesized according to the K562 DNA sequence, which amplified 1.2 kb of 5'HS5 LTR including 130 bases of genomic DNA downstream of the LTR (see Methods and Figure 2).

The PCR results indicate that the 5'HS5 ERV-9 LTR is conserved in the genomes of the gorilla and people across racial lines. Fifteen out of a total of 17 human DNAs isolated from Africans, Arab, Asian and Caucasians and from human cell lines K562 and HL60 produced amplicons of the anticipated length of 1.2 kb. However, two of the nine African DNAs

produced either a shorter amplicon of 1.1 kb or both a longer 1.4 kb and a shorter 1.1 kb amplicons, while the gorilla DNA produced an even shorter amplicon of 0.9 kb (Figure 6).

It was possible that the observed amplicons might be spurious PCR products amplified by the primer pair from other ERV-9 LTRs in the human or the gorilla genome, since the 5' primer was located within the U3 region immediately upstream of the enhancer repeats—a region present also in some of the other ERV-9 LTRs even though the 3' primer was located in the unique genomic DNA region (see Figure 2). Therefore, the authenticity of the amplicons was further confirmed by DNA sequencing. Four standard amplicons of 1.2 kb from two Caucasian and two African DNAs, two shorter amplicons of 1.1 kb from the African DNAs, and the 0.9 kb amplicon of the gorilla DNA were sequenced (Figure 5). The electropherograms of the DNA sequences showed sharp DNA sequence ladders with only a couple of ambiguities where two different bases occupied the same sequence positions, indicating that the two homologous chromosomes contained base polymorphism at these positions. All the sequenced amplicons showed base identities of 98-99% in both the LTR and the 3' flanking genomic DNA; the only exception was the fewer number of U3 repeats in some people and in the gorilla (Figures 5 and 6). If the sequenced amplicons contained amplification products generated also from other homologous ERV-9 LTRs, the electropherograms would have contained too many sequence ambiguities to generate clearly readable sequences. The above observations indicate that the amplicons were genuine products of the 5'HS5 LTR in the human and gorilla genomes.

In both the shorter human amplicons containing eleven U3 repeats, the deletion of three complete U3 repeats was generated apparently by the same in phase deletion event so the subtype organizations of both amplicons were identical, 1-2-3-4-1-2-3-4-1-2-1, (Figures 5 and 6). In the gorilla amplicon with five U3 repeats, the subtype organization is 1-2-3-4-1 (Figures 5 and 6). The apparent genomic insertion site of the LTR—the GTAT sequence is conserved in both the human and gorilla amplicons (Figure 5).

The remarkable sequence identities in the 5'HS5 LTR between human and gorilla and among people of different races indicate that this LTR was probably inserted into the 5' boundary area of the β -globin LCR at least 10 million years ago before the divergence of the human and apes and it has
5 been conserved in the genomes of the higher primates during the ensuing years of evolution. These observations indicate that this 5'HS5 LTR-retrotransposon is likely conserved for the preservation of a relevant cellular function of the host.

The 5'HS5 LTR ERV-9 LTR possesses enhancer and promoter activities in erythroid cells: To demonstrate that the enhancer and promoter regions in the 5'HS5 LTR possess enhancer and promoter activities, seven recombinant CAT plasmids were made (Figure 7). LTR-CAT (Construct 1) contained the 1 kb LTR spanning the 14 U3 enhancer repeats, U3 promoter, R and U5 spliced 5' of the CAT gene in the absence of a promoter in the
10 vector. To determine whether other regions of the 5' boundary area of the LCR also possessed enhancer and promoter activities, the control Ups-CAT plasmid (Construct 2) contained a 1 kb DNA (Ups) located further upstream of the LTR (Figure 1). The HS2- ϵ p-CAT plasmid (Construct 4) that
15 contained the strong HS2 enhancer of the LCR (Tuan et al, 1989) coupled to the ϵ -globin promoter (ϵ p) served as the standard with which to compare the enhancer and promoter activities of the 5'HS5 LTR. To test if the enhancer in 5'HS5 LTR can synergize with and activate the HS5 site located naturally downstream of and proximal to the LTR, LTR- ϵ p-CAT, HS5- ϵ p-CAT and LTR-HS5- ϵ p-CAT (Constructs 5, 6 and Figure 7) contained respectively the
20 LTR and HS5 site spliced either separately or together into ϵ p-CAT (Construct 3). The plasmids were transiently transfected into erythroid K562 and MEL cells and nonerythroid HL60 cells and stably integrated into K562 cells.

Transient transfection results indicate that in human erythroid K562
30 cells, the LTR in LTR-CAT plasmid displayed enhancer and promoter activities that were approximately 50% of the combination of the HS2 enhancer and the ϵ -globin promoter in the HS2- ϵ p-CAT plasmid. In

contrast, in murine erythroid MEL cells and human nonerythroid HL60 cells, both LTR-CAT and HS2- ϵ p-CAT displayed much lower enhancer and promoter activities (Figure 8). The low enhancer activity of the HS2 enhancer in MEL cells was due apparently to the inactivity of the cis-linked embryonic ϵ -globin promoter in MEL cells expressing the adult globin program; when linked to the more permissive adult β -globin promoter, the HS2 enhancer displayed much higher enhancer activity in MEL cells (Cavallesco and Tuan, 1997). Likewise, the U3 enhancer in the LTR may also be potentially active in MEL cells; its apparently low enhancer activity may be due to the low activity in MEL cells of the U3 promoter which shares certain sequence identities with the ϵ -globin promoter (Figure 4).

When stably integrated into the genome of K562 cells, the LTR displayed enhancer and promoter activities that were approximately 30% of those of the HS2- ϵ p-CAT plasmid (Figure 9). However, in integrated LTR-HS5- ϵ p-CAT plasmid, the LTR enhancer synergized with the HS5 site and activated the CAT gene to a level comparable to that displayed by the HS2 enhancer in HS2- ϵ p-CAT (Figure 9). These results indicate that the 5'HS5 LTR possesses enhancer and promoter activities in erythroid cells and it synergized with and activated the HS5 site.

The endogenous 5'HS5 LTR activates the transcription of downstream DNA preferentially in erythroid cells: It was next determined if the endogenous 5'HS5 LTR also exhibits enhancer and promoter activities and can activate the transcription of the downstream R region and the flanking genomic DNA in the β -globin LCR. The transcriptional statuses of the 5'HS5 LTR and downstream genomic DNA were determined by RT-PCR in erythroid K562 and non-erythroid T-lymphocytes and placental cells.

Four PCR primer pairs were made (Figure 10). Primer pair 1 was synthesized to determine if the entire LTR between the U3 enhancer and the U5 regions as well as the genomic DNA immediately downstream of it was transcribed. Primer pair 2 was synthesized to detect retroviral mRNA transcripts of the R and U5 regions whose synthesis was activated by the U3

enhancer and promoter. In order to ensure that Primer pair 2 detected the RNA transcribed specifically from the 5'HS5 LTR and not RNAs transcribed from other ERV-9 LTRs, the forward Primer was located in the R region that contains a number of polymorphic bases among the ERV-9 LTRs Figure 2; Henthorn et al, 1986 and Lania et al, 1992) and the reverse primer is located in the genomic DNA immediately downstream of the LTR. Primer pairs 3 and 4 were synthesized to confirm that the RNAs detected by Primer pairs 1 and 2 were indeed transcribed from 5'HS5 ERV-9 LTR. These two primer pairs contained the same two respective forward primers as Primer pairs 1 and 2 but shared a common reverse primer located in the genomic DNA 110 bases further downstream of the reverse primer of Primer pairs 1 and 2. Hence, the authentic RT-PCR bands of the 5'HS5 LTR generated by these primer pairs would be 110 bases longer than those generated respectively by Primer pairs 1 and 2 (Figure 10).

Consistent with the design of the primer pairs (Figure 10), the sizes of the RT-PCR bands produced by Primer pairs 3 and 4 were indeed longer by 110 bases than those produced by Primer pairs 1 and 2. This indicates that the RT-PCR bands generated by Primer pairs 1-4 were genuine products amplified from the 5'HS5 LTR and not from other ERV-9 LTRs in the human genome. In addition, the authenticity of the PCR band produced by Primer pair 3 had been confirmed by direct DNA sequencing (Figure 5).

For a semi-quantitative comparison of the intensities of RT-PCR bands generated by primer pairs 1-4 in different RNA samples, a β -actin primer pair spanning a region in the ubiquitous β -actin mRNA assumed to be expressed at a constant level in different cell types was included in the RT-PCRs. Consistent with this assumption, the intensities of the β -actin band generated by the same amount of different RNAs were similar. The relative intensities of the LTR bands with respect to the intensity of the β -actin band generated from aliquots of the same cDNA master stock as the LTR bands (see Methods) were then compared.

The RT-PCR results indicate that the endogenous 5'HS5 LTR promoted the transcription of the R and U5 regions. In both erythroid and nonerythroid cells, Primer pairs 2 and 4 generated amplification bands of the

R and U5 regions. However, the LTR enhancer and promoter appear to be more active in erythroid than in nonerythroid cells, as the amplification bands generated from RNAs of K562 cells and CFU-E were relatively stronger than those of nonerythroid T-lymphocytes, N-Tera and HL60 cells.

5 An apparent exception to the above observation was the nonerythroid placenta which also generated strong LTR bands. This may be due to contamination in placenta of abundant maternal and fetal blood erythroid cells in which the 5'HS5 LTR enhancer and promoter were active. On the other hand, the 5'HS5 LTR enhancer and promoter may also be active in the
10 placenta since many HERV's and their solitary LTRs have been found to be capable of initiating viral RNA synthesis from the R region in placental cells (Wilkinson et al, 1994; Lower et al 1996).

Further upstream of the R region in the LTR, no additional transcriptional initiation sites appear to exist in the majority of the cell types
15 tested, since Primer pairs 1 and 3 did not generate detectable bands from RNAs of erythroid K562 and nonerythroid T-lymphocytes, N-Tera and HL60 cells. However, Primer pairs 1 and 3 generated faint amplification bands from erythroid CFU-E and nonerythroid placenta RNAs. This suggests that CFU-E and placenta may contain additional transcriptional
20 initiation sites proximal to the 5'HS5 LTR.

The above RT-PCR results indicate that the endogenous 5'HS5 LTR possesses apparent enhancer and promoter activities and is capable of promoting the transcription of the R and U5 regions in the LTR and of further downstream genomic DNA in the LCR.

25 **DISCUSSION**

This example shows that a solitary ERV-9 LTR with the characteristics of a retrotransposon is located proximal to the HS5 site in the apparent 5' boundary area of the β -globin LCR. This 5' HS5 ERV-9 LTR possesses unusual sequence features in the multiple tandem repeats of the U3
30 enhancer region. The U3 repeats and the immediately downstream U3 promoter contain within 700 DNA bases nine GATA, four CACCC and ten CCAAT sites. These DNA motifs can bind respectively to the cognate GATA (Orkin, 1992) and CACCC (Miller and Bieker, 1993; Crossley et al,

1996) transcription factors expressed abundantly in erythroid cells and to the CCAAT factors C/EBP (Johnson and McKnight, 1989) and NF-Y (Bi et al, 1997), expressed in many hematopoietic and nonhematopoietic cells. The high concentration of these motifs in the U3 region suggests that the 5'HS5
5 ERV-9 LTR may be preferentially active in erythroid cells.

The 5'HS5 LTR is conserved in the gorilla and in people of different racial lineages, indicating that this LTR was probably inserted into its location at the 5' boundary area of the LCR before species divergence between human and gorilla approximately 10 million years ago. The
10 conservation of the 5'HS5 LTR during evolution of the higher primates suggests that this LTR-retrotransposon may serve a relevant cellular function of the host.

Functional tests with the CAT reporter gene assays show that the 5'HS5 LTR, in line with its component sequence motifs, possesses enhancer
15 and promoter activities preferentially in erythroid cells. Moreover, the LTR enhancer activity can synergize with and activate the cis-linked HS5 site in the LCR.

REFERENCES

- 20 Anagnou, N.P., Perez-Stable, C., Gelinas, R., Constantini, F., Liapaki, K., Constantopoulou, M., Kosteas, T., Moschonas, N.K., and Stamatoyannopoulos, G. (1995). Sequences Located 3' to the Breakpoint of the Hereditary Persistence of Fetal Hemoglobin-3 Deletion Exhibit Enhancer Activity and Can Modify the Developmental Expression of the Human Fetal
25 α -Globin Gene in Transgenic Mice. *J. Biol. Chem* **270**:10256-10263.
- Ashe, H., Monks, J., Wijgerde, M., Fraser, P. and Proudfoot, N. (1997). Intergenic transcription and transinduction of the human β -globin locus. *Genes & Dev.* **11**, 2494-2509.
- Baralle, F., Shoulders, C., and Proudfoot, N. (1980). The primary
30 structure of the human ϵ -globin gene. *Cell* **21**, 621-626.
- Bi, W., Wu, L., Coustry, F., Crombrughe, B. and Maity, S. (1997). DNA binding specificity of the CCAAT-binding factor CBF/NF-Y. *J. Biol. Chem.* **272**, 26562-26572.

- Cavallesco, R. and Tuan, D. (1997). Modulatory subdomains of the HS2 enhance differentially regulate enhancer activity in erythroid cells at different developmental stages. *Blood Cells, Molecules and Diseases* **23**, 8-26. (World-wide web URL: <http://www.scripps.edu/bcmd>).
- 5 Chung, J.H., Whiteley, M. & Felsenfeld, G. (1993). A 5' element in the chicken β -globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* **74**, 505-514.
- Coffin, J. (1984). Endogenous retroviruses. In "RNA tumor viruses" (R. Weiss, N. Teich, H. Varmus, and J. Coffin, eds), Vol. 1, pp. 1109-1203, Cold Spring Harbor Laboratory, N.Y.
- 10 Crossley, M., Whitelaw, E., Perkins, A., Williams, G., Fujiwara, Y., and Orkin, S. (1996). Isolation and characterization of the cDNA encoding BKLf/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. *Mol. Cell. Biol.* **16**, 1695-1705.
- 15 Dhar, V., Nandi, A., Schildkraut, C.L., & Skoultschi, A.I. (1990). Erythroid-specific nuclease-hypersensitive sites flanking the human β -globin domain. *Mol. Cell. Biol.* **10**, 4324-4333.
- DiCristofano, A., Strazzullo, M., Longo, L., and LaMantia, G. (1995). Characterization and genomic mapping of the ZNF80 locus: expression of this zinc-finger gene is driven by a solitary LTR of ERV9 endogenous retroviral family. *Nucleic Acids Research* **23**, 2823-2830.
- 20 DiCristofano, A., Strazzullo, M., Longo, L., and LaMantia, G. (1995). Mobilization of an ERV9 Human Endogenous Retroviral Element during Primate Evolution. *Virology* **213**, 271-275.
- 25 Dolittle, R. and Sapienza, C. (1980). Selfish genes, the phenotype paradigm and genomic evolution. *Nature* **284**, 601-603.
- Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., DeRiel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C., & Proudfoot, N.J. (1980). The structure and evolution of the human β -globin gene family. *Cell* **21**, 653-668.
- 30 Fan, H. (1994). Retroviruses and their role in cancer. In "The Retroviridae" (J. Levy ed), Vol. 3, pp. 313-362, Plenum Press, N.Y.

- Feuchter, A.E., Freeman, J.D., and Mager, D.L. (1992). Strategy for Detecting Cellular Transcripts Promoted by Human Endogenous Long Terminal Repeats: Identification of a Novel Gene (CDC4Lease) with Homology to Yeast CDC4. *Genomics* **13**, 1237-1246.
- 5 Feuchter-Murthy, A.E., Freeman, D.J., and Mager, D.L. (1993) Splicing of a human endogenous retrovirus to a novel phospholipase A2 related gene. *Nucleic Acids Research* **21**, 135-143.
- Forrester, W.C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G., & Groudine, M. (1987). Evidence for a locus
10 activating region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucl. Acids Res.* **15**, 10159-10177.
- Golemis, E.A., Speck, N.A., and Hopkins, N. (1990). Alignment of U3 Region Sequences of Mammalian Type C Viruses: Identification of highly conserved motifs and implications for enhancer design. *Journal of*
15 *Virology* **64**:534-542.
- Goodchild, N., Wilkinson, D.A., and Mager, D.L. (1992). Human endogenous long terminal repeat provides a polyadenylation signal a novel, alternatively spliced transcript in normal placenta. *Virology* **21** 287-294.
- Grosveld, F., Assendelft, G.B.V., Greaves, D.R., & Kollias, G.
20 (1987). Position-independent, high-level expression of the human β -globin gene in transgenic mice. *Cell* **51**, 975-985.
- Hardison, R., Slightom, J., Gumucio, D., Goodman, M. Stojanovic, N. and Miller, W. (1997). Locus Control Regions of mammalian β -globin gene cluster: combining phylogenetic analyses and experimental results to
25 gain functional insights. *Gene* **205**: 73-94.
- Henikoff, S., Greene, E., Pietrokovski, S., Bork, P., Attwood, T. and Hood, L. (1997). Gene families: The taxonomy of protein paralogs and chimeras. *Science* **278**, 609-614.
- Henthorn, P., Mager, D., Huisman, H. and Smithies, O. (1986). A
30 gene deletion ending within a complex array of repeated sequences 3' to the human β -globin gene cluster. *Proc. Natl. Acad. Sci. U.S.A* **83**, 5194-5198.
- Jarman, A.P. & Higgs, D.R. (1988). Nuclear scaffold attachment sites in the human globin gene complexes. *EMBO J* **7**, 3337-3344.

- Johnson, P. and McKnight, S. (1989). Eukaryotic transcriptional regulatory proteins. *Annual Rev. Biochem.* **58**, 799-739.
- Kellum, R. & Schedl. P. (1991). A position-effect assay for boundaries of higher order chromosomal domains. *Cell*, **64**, 941-950.
- 5 Keshet, E., Schkff. R., Itin, A. (1991). Mouse retrotransposons: a cellular reservoir of long terminal repeat (LTR) elements with diverse transcriptional specificities. *Adv. Cancer Res.* **56**, 215-251.
- Ko, L.J., & Engel, J.D. (1993). DNA-binding specificities of the GATA transcription factor family. *Mol. Cell. Biol.* **13**, 4011-4022.
- 10 Kong, S., Bohl, D., Li. C. and Tuan, D. (1997). Transcription of the HS2 enhancer toward a cis-linked gene is independent of the orientation, position, and distance of the enhancer relative to the gene. *Mol. Cell. Biol.* **17**, 3955-3965.
- LaMantia, G. Pengue, G., Maglione, D., Pannuti, A., Pascucci, A., and Lania, L. (1989). Identification of new human repetitive sequences: characterization of the corresponding cDNAs and their expression in embryonal carcinoma cells. *Nucleic Acids Research* **17**: 5913-5920.
- 15 LaMantia, G., Maglione, D., Pengue, G., DiCristofano, A., Simeone, A., Lanfrancone, L., and Lania, L. (1991). Identification and characterization of novel human endogenous retroviral sequences preferentially expressed in undifferentiated embryonal carcinoma cells. *Nucleic Acids Research* **19**, 1513-1520.
- LaMantia, G., Majello, B., DiCristofano, A., Strazzullo, M., Minchiotti, G., and Lania, L. (1992). Identification of regulatory elements within the minimal promoter region of the human endogenous ERV9 proviruses: accurate transcription initiation is controlled by an Inr-like element. *Nucleic Acids Research* **20**, 4129-4136.
- 25 Lania, L., Di Cristofano, A., Strazzullo, M., Pengue, G., Majello, B., and LaMantia, G. (1992). Structural and functional Organization of the Human Endogenous Retroviral ERV9 Sequences. *Virology* **191**, 464-468.
- 30 Lenz, J., Celander, D., Crowther, R.L., Patarca, R., Perkins, D.W., & Haseltine, W.A. (1984). Determination of the leukaemogenicity of a murine

- retrovirus by sequences within the long terminal repeat. *Nature* **308**: 467-470.
- Li, Q., Powers, P.A., and Smithies, O. (1985). Nucleotide sequence of 16 kilobase pairs of DNA 5' to the human β -globin gene. *J. Biol. Chem.* **260**, 14901-14910.
- Li, Q., and Stamatoyannopoulos, G. (1995). Hypersensitive site 5 of the human β Locus Control Region functions as a chromatin insulator. *Blood* **84**, 1399-1401.
- Long, Q., Li, C., Nechtman, J., Tjia, J., Yoo, J. and Taun, D. (1995). Nucleotide sequence and transcriptional analysis of DNA upstream of hypersensitive site 4 in the human β -globin locus control region. *Blood* **86**, 472a.
- Lower, R., Lower, J., and Kurth, R. (1996). The viruses in all of us: Characteristics and biological significance of human endogenous retroviruses. *Proc. Natl. Acad. Sci. USA* **93**, 5177-5184.
- Ludwig, D., Dhen, F., Peterson, S., Nussenzweig, A., Li, G., and Chen, D. (1997). Ku80 gene expression is Sp1-dependent and sensitive to CpG methylation within a novel cis element. *Gene* **199**, 181-194.
- Merika, M., & Orkin, S.H., (1993). DNA-binding specificity of GATA family transcription factors. *Mol Cell. Biol.* **13**, 3999-4010.
- Miller, I. and Bieker, J. (1993). A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the *Kruppel* family of nuclear proteins. *Mol. Cell. Biol.* **13**, 1776-2786.
- Nienhuis, A., Anagnou, N. and Ley, T. (1984). Advances in thalassemia research. *Blood* **63**, 738-758.
- Orkin, S.H. (1992). GATA-binding transcription factors in hematopoietic cells. *Blood* **80**, 575-581.
- Poncz, M., Sowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E. and Surrey, S. (1982). Construction of gene libraries from small amounts of peripheral blood. *Hemoglobin* **6**, 27-36.
- Poncz, M., Schwartz, E., Ballantine, M. and Surrey, S. (1983). Nucleotide sequence analysis of the $\delta\beta$ -globin gene region in human. *J. Biol. Chem.* **258**, 11599-11609.

- Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Schulte, A.M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A.T., and Wellstein, A. (1996). Human trophoblast and choriocarcinoma expression of
5 the growth factor pleiotrophin attributable to germ-line insertion of an endogenous retrovirus. *Proc. Natl. Acad. Sci. USA* **93**, 14759-14764.
- Shen, S., Slighthom, J. and Smithies, O. (1981). A history of the human fetal globin gene duplication. *Cell*. **26**, 191-203.
- Sibley, C. and Ahlquist, J. (1987). DNA hybridization evidence of
10 hominoid phylogeny: Results from an expanded data set. *J. Mol. Evol.* **26**, 99-121.
- Smit, A. (1996). The origin of interspersed repeats in the human genome. *Current Opinion in Genetics and Development*. **6**, 743-748.
- Speck, N.A., Renjifo, B., Golemis, E., Frederickson, T.N., Hartley, J.W., and
15 Hopkins, N. (1990). Mutation of the core or adjacent LVb elements of the Moloney murine leukemia virus enhancer alters disease specificity. *Genes & Development* **4**, 233-242.
- Stavenhagen, J.B. and Robins, D.M. (1988) An ancient provirus has imposed androgen regulation on the adjacent mouse sex-limited protein
20 gene. *Cell* **55**247-254.
- Steif, A., Winter, D.M., Stratling, W.H. & Sippel, A.E. (1989). A nuclear DNA-attachment element mediates elevated and position-independent gene activity. *Nature* **341**, 343-345.
- Starzzullo, M., Majello, B., Lania, L., and LaMantia, G. (1994).
25 Mutational Analysis of the Human Endogenous ERV9 Provirus Promoter Region. *Virology* **200**, 686-695.
- Strazullo, M., Parisi, T., DiCristofano, A., Rocchi, M., LaMantia, G. (1998). Characterization and genomic mapping of chimeric ERV9 endogenous retroviruses-host gene transcripts. *Gene* **206**, 77-83.
- 30 Suzuki, H., Hosokawa, Y., Toda, H., Nishikim, M., and Ozawa, T. (1990). Common Protein-binding Sites in the 5'-Flanking Regions of Human Genes for Cytochrome c1 and Ubiquitine-binding Protein. *J. Biol. Chem.* **265**, 8159-8163.

- Temin, H. (1981) Structure, variation and synthesis of retrovirus long terminal repeat. *Cell* **27**, 1-3.
- Ting, C., Rosenberg, M., Snow, C., Samuelson, L., and Meisler, M. (1992). Endogenous retroviral sequences are required for tissue-specific
5 expression of a human salivary amylase gene. *Genes & Dev.* **6**, 1457-1465.
- Tuan, D., Solomon, W., Li, Q., & London, I.M. (1985). The β -globin gene domain in human erythroid cells. *Proc. Natl. Acad. Sci USA* **82**, 6384-6388.
- 10 Tuan, D., Solomon, W.B., London, I.M. & Lee, D.P. (1989). An erythroid-specific, developmental-stage-independent enhancer far upstream of the human β -like globin genes. *Proc. Natl. Acad. Sci. USA* **86**, 2554-2558.
- 15 Tuan, D., Oh, Y.D., Venditti, C., Cavellesco, R., LeBoulch, P., Huang, G. and London, I. (1990). A distant erythroid enhancer in the regulation of human globin genes during erythropoiesis. In "Molecular Biology of Hematopoiesis (N.G. Abraham, G. Konwalinka, L. Sachs and C.G. Wiedermann, eds), Intercept Ltd., England.
- 20 Tuan, D., Kong, S., & Hu, K. (1992). Transcription of the hypersensitive site HS 2 enhancer in erythroid cells. *Proc. Natl. Acad. Sci USA* **89**, 11219-11223.
- Vogel, F. and Motulsky, A. (1986). Human Genetics. pp. 534-538. Publisher: Springer Verlag.
- 25 Weber-Benarous, A., Cone, R., London, I. and Mulligan, R. (1988). Retroviral-mediated transfer and expression of human β -globin genes in cultured murine and human erythroid cells. *J. Biol. Chem.* **263**, 6142-6145.
- Wickrema, A., Krantz, S., Winklemann, J. and Bondurant, M. (1992). Differentiation and erythroid expression in human erythroid progenitor cells. *Blood* **80**, 1940-46.
- 30 Wilkison, D., Mager, D. and Leong, J. (1994). Endogenous human retroviruses. In "The retroviridae" (J. Levy ed), Vol 3. pp. 465-535, Plenum Press, N.Y.
- Yang, R., Fristensky, B., Deutch, A.H., Huang, R.C., Tan, Y.H., Narang, S.A. & Wu, R. (1983). The nucleotide sequence of a new human

repetitive DNA consists of eight tandem repeats of 66 base pairs. *Gene* 25:59-66.

Yu, J., Bock, J.H., Slightom, J.L. & Villeponteau, B. (1994). A 5' β -globin matrix-attachment region and the polyoma enhancer together confer position-independent transcription. *Gene* 139, 139-145.

Zucchi, I. and Schlessinger, D. (1992). Distribution of moderately repetitive sequences pTR5 and LF1 in Xq24-q28 human DNA and their use in assembling YAC contigs. *Genomics* 12, 264-275.

10 It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to "the antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

15 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, 20 and materials are as described.

CLAIMS

1. A nucleic acid molecule comprising all or a functional portion of the U3 enhancer (nucleotides 595 to 1193 of SEQ ID NO:1), wherein a functional portion is a portion of the U3 enhancer that retains enhancer function.
2. The nucleic acid molecule of claim 1 further comprising all or a functional portion of the U3 insulator (nucleotides 5 to 594 of SEQ ID NO:1) operably linked to the enhancer, wherein a functional portion of the U3 insulator is a portion of the U3 insulator that retains insulator function.
3. The nucleic acid molecule of claim 1 further comprising all or a functional portion of the U3 promoter (nucleotides 1194 to 1322 of SEQ ID NO:1) operably linked to the enhancer, wherein a functional portion of the U3 promoter is a portion of the U3 promoter that retains promoter function.
4. The nucleic acid molecule of claim 1 further comprising the U3 R region (nucleotides 1322 to 1380 of SEQ ID NO:1) operably linked to the enhancer.
5. The nucleic acid molecule of claim 1 further comprising a gene operably linked to the enhancer.
6. The nucleic acid molecule of claim 2 wherein the gene encodes a protein.
7. A vector comprising the nucleic acid molecule of claim 6.
8. A vector comprising the nucleic acid molecule of claim 5.
9. The vector of claim 8 wherein the vector is a retroviral vector.
10. A cell transformed with the vector of claim 8.
11. The cell of claim 10 wherein the cell is a mammalian cell.
12. The cell of claim 11 wherein the cell is a cell in an animal.
13. A method of expressing a protein, the method comprising culturing the transformed cell of claim 7, wherein the protein encoded by the protein encoded by the gene is expressed.
14. A method of expressing a gene in an animal, the method comprising introducing the transformed cell of claim 10 into an animal, wherein the gene is expressed.

15. A method of expressing a gene in an animal, the method comprising introducing the vector of claim 8 into cells of an animal, wherein the gene is expressed.

16. A nucleic acid molecule comprising all or a functional portion of the U3 insulator (nucleotides 5 to 594 of SEQ ID NO:1), wherein a functional portion is a portion of the U3 insulator that retains insulator function.

17. The nucleic acid molecule of claim 16 further comprising all or a functional portion of the U3 promoter (nucleotides 1194 to 1322 of SEQ ID NO:1) operably linked to the insulator, wherein a functional portion of the U3 promoter is a portion of the U3 promoter that retains promoter function.

18. The nucleic acid molecule of claim 16 further comprising the U3 R region (nucleotides 1322 to 1380 of SEQ ID NO:1) operably linked to the insulator.

19. The nucleic acid molecule of claim 16 further comprising a gene operably linked to the insulator.

20. The nucleic acid molecule of claim 19 wherein the gene encodes a protein.

21. A vector comprising the nucleic acid molecule of claim 20.

22. A vector comprising the nucleic acid molecule of claim 19.

23. The vector of claim 22 wherein the vector is a retroviral vector.

24. A cell transformed with the vector of claim 22.

25. The cell of claim 24 wherein the cell is a mammalian cell.

26. The cell of claim 25 wherein the cell is a cell in an animal.

27. A method of expressing a protein, the method comprising culturing the transformed cell of claim 21, wherein the protein encoded by the protein encoded by the gene is expressed.

28. A method of expressing a gene in an animal, the method comprising introducing the transformed cell of claim 24 into an animal, wherein the gene is expressed.

29. A method of expressing a gene in an animal, the method comprising introducing the vector of claim 22 into cells of an animal, wherein the gene is expressed.

30. A nucleic acid molecule comprising a modified U3 enhancer, wherein one or more of the repeat units of the enhancer are deleted, one or more of the repeat units are replaced with a repeat unit of the enhancer having a different sequence than the repeat unit that is replaced, one or more repeat units of the enhancer are added to the enhancer, or a combination of one or more of these modifications,

wherein the modified enhancer retains enhancer function.

31. A nucleic acid molecule comprising an enhancer, wherein the enhancer has three or more repeats, wherein each repeat has one of the following sequences: TRTCTAGCTCADGGTTTGTRAAYRCACCAATCAGCACTCTG (SEQ ID NO:12),

TATCTAGCTCAGGGATTGTAAATACACCAATCGGCAGTCTG (SEQ ID NO:8),

TGTCTAGCTCAAGGTTTGTAAACACACCAATCAGCACCTG (SEQ ID NO:9),

TATCTAGCTCAGGGTTTGTGAATGCACCAATCAACACTCTG (SEQ ID NO:10),

TGTCTAGCTACTCTGTGGGGACGTGGAGAACCTTTA (SEQ ID NO:11).

32. The nucleic acid molecule of claim 31, wherein each repeat has one of the following sequences:

TATCTAGCTCAGGGATTGTAAATACACCAATCGGCAGTCTG (SEQ ID NO:8),

TGTCTAGCTCAAGGTTTGTAAACACACCAATCAGCACCTG (SEQ ID NO:9),

TATCTAGCTCAGGGTTTGTGAATGCACCAATCAACACTCTG (SEQ ID NO:10),

TGTCTAGCTACTCTGTGGGGACGTGGAGAACCTTTA (SEQ ID NO:11).

33. The nucleic acid molecule of claim 31, wherein each repeat has the following sequence:

TRTCTAGCTCADGGTTTGTRAAYRCACCAATCAGCACTCTG (SEQ ID NO:12).

34. The nucleic acid molecule of claim 31 wherein the enhancer has from three to fourteen repeat units.

35. A nucleic acid molecule comprising an enhancer, wherein the enhancer is a primate 5' HS5 ERV-9 LTR enhancer.

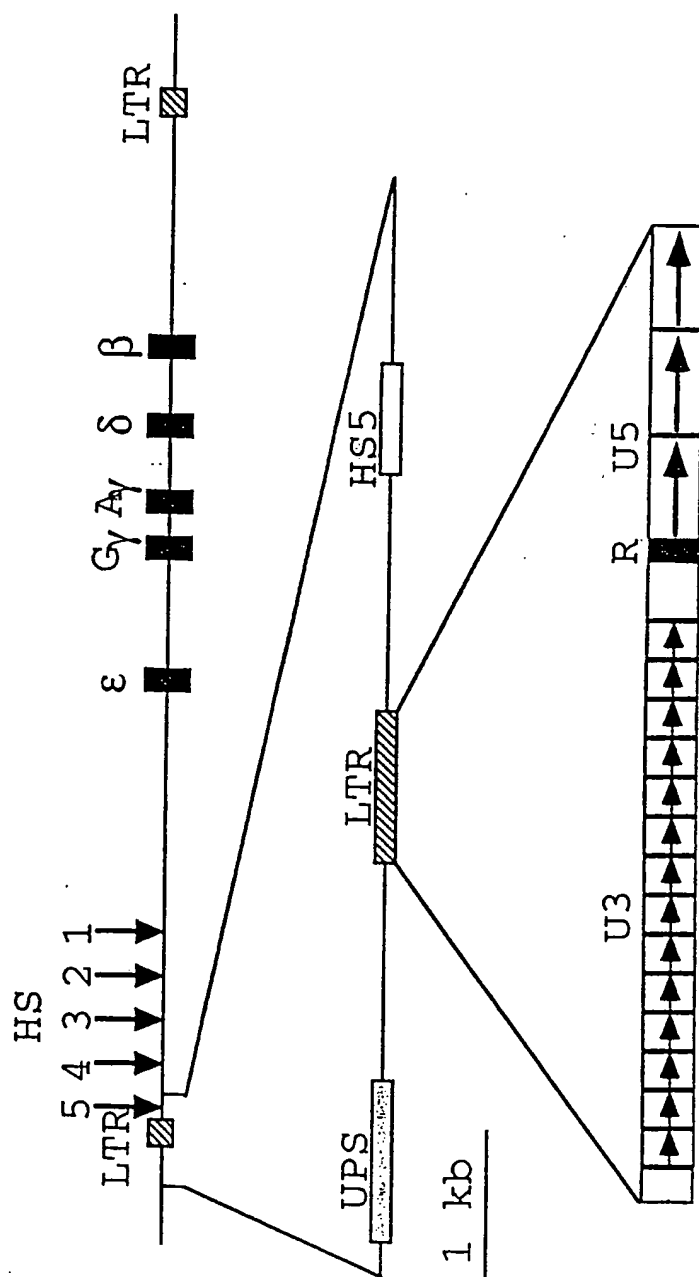


Figure 1

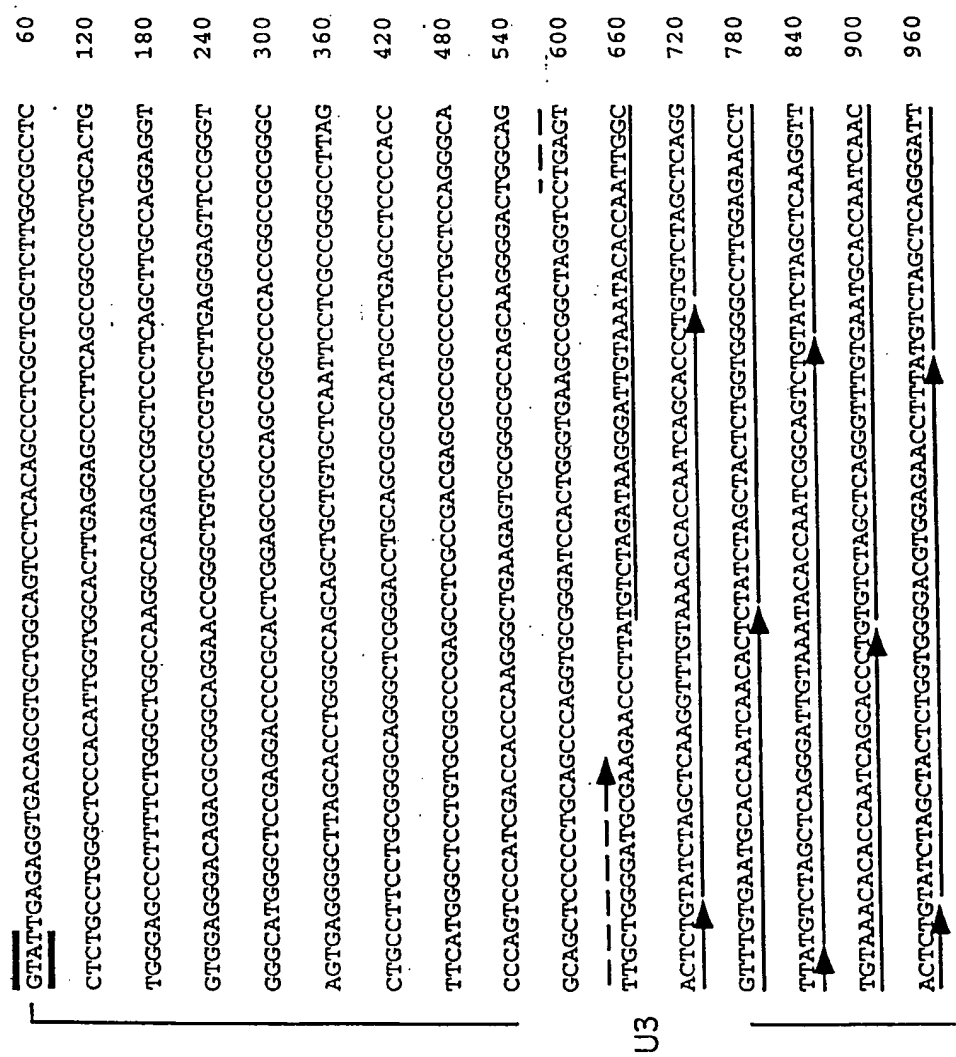


Figure 2A

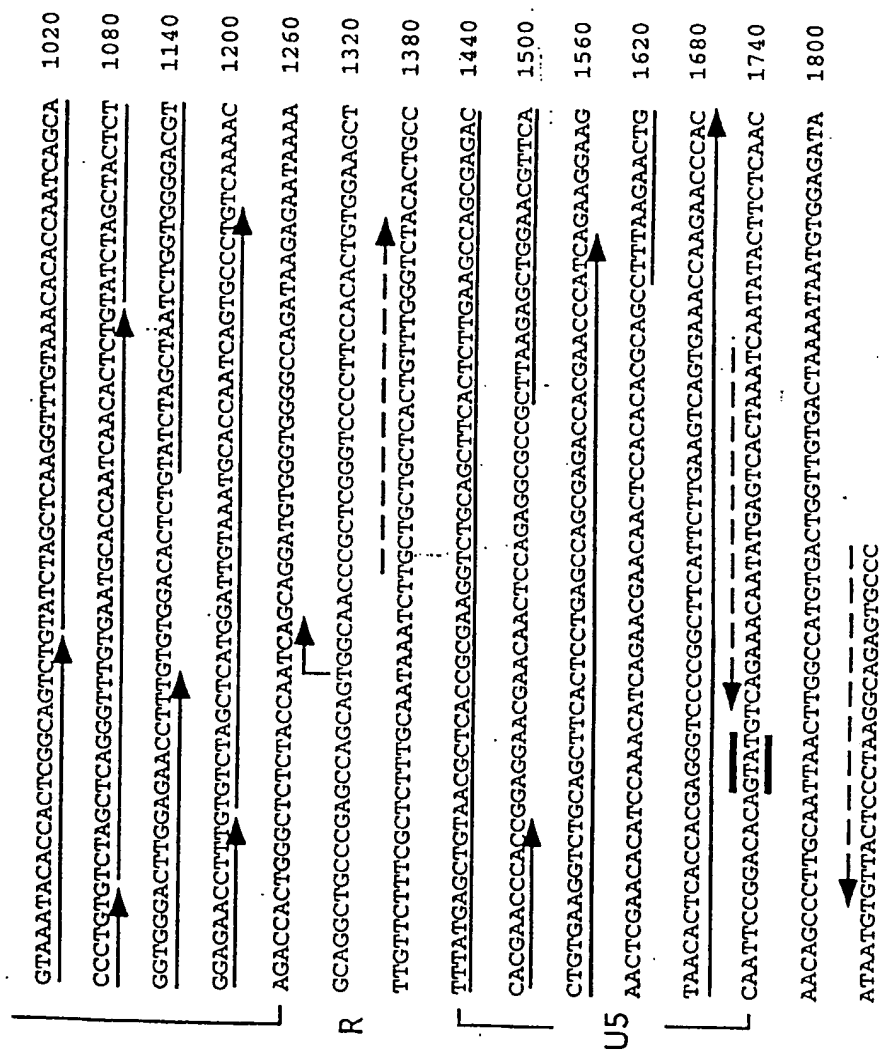


Figure 2B

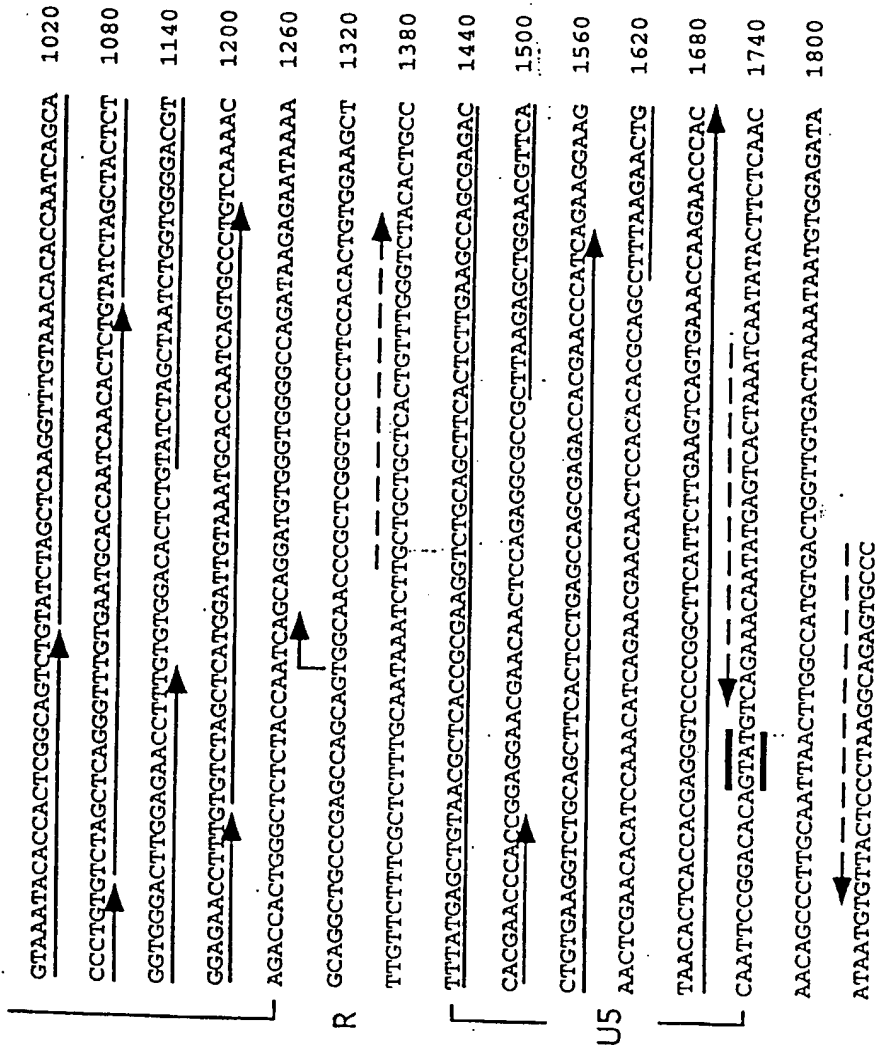
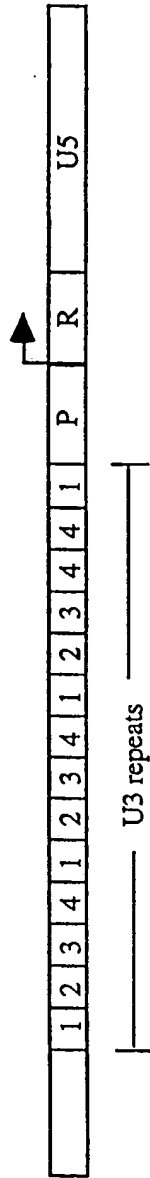


Figure 2B



U3 repeat subtypes:

1. ^{GATA} TATCTAGCTC AGGGAATTGTA AATACACCAA ^{CCAAT} TCGGCAGTCT G
CACCC
2. TGTCTAGCTC AAGGTTTGTG AACACACCAA TCAGCACCT G
3. TATCTAGCTC AGGTTTGTG AATGCACCAA TCAACACTCT G
CCACC
4. TGTCTAGCTA CTCTGGTGGG GACGTGGAGA ACCTTTA

Consensus sequences of U3 Repeats:

5'HS5 ^{GATA} Tg/aICTAGCT C Ag/taGG T TTGTa/gAAc/t a/gCACCAATCAGCACTCTG ^{CCAAT}

3'β T G TCTAGCTa/ca A GG T TTGT A AA T G CACCAATCAGCACTCTG

LTR2 Tg/aICTAGCTcaA g/a GGa/tTTGT A AA C a/gCACCAATCAGCACTCTG

Figure 3

U3 and ε-globin Promoter sequences:

5'HS5 TCAAAACAGA CCACTGGGCT CTCTACCAAT CAGCAGGATG TGGTGGGGC CACCC

GATA
CAGATAAGAG AATAAAGCA GGCTGCCCGA GCCAGCAGTG GCAA

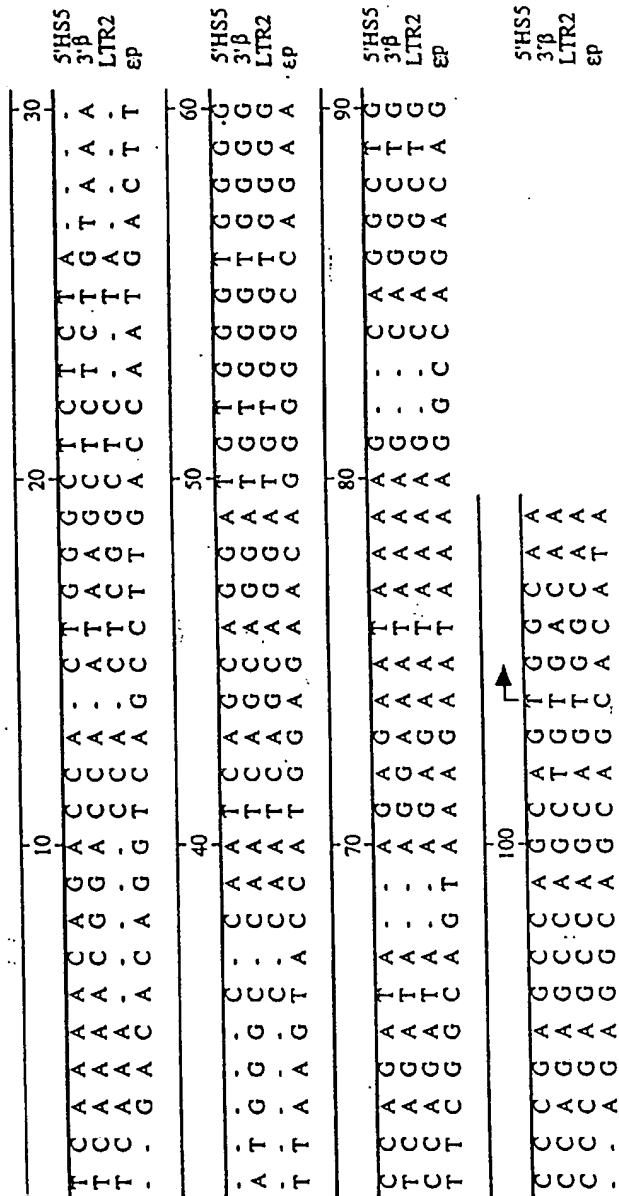


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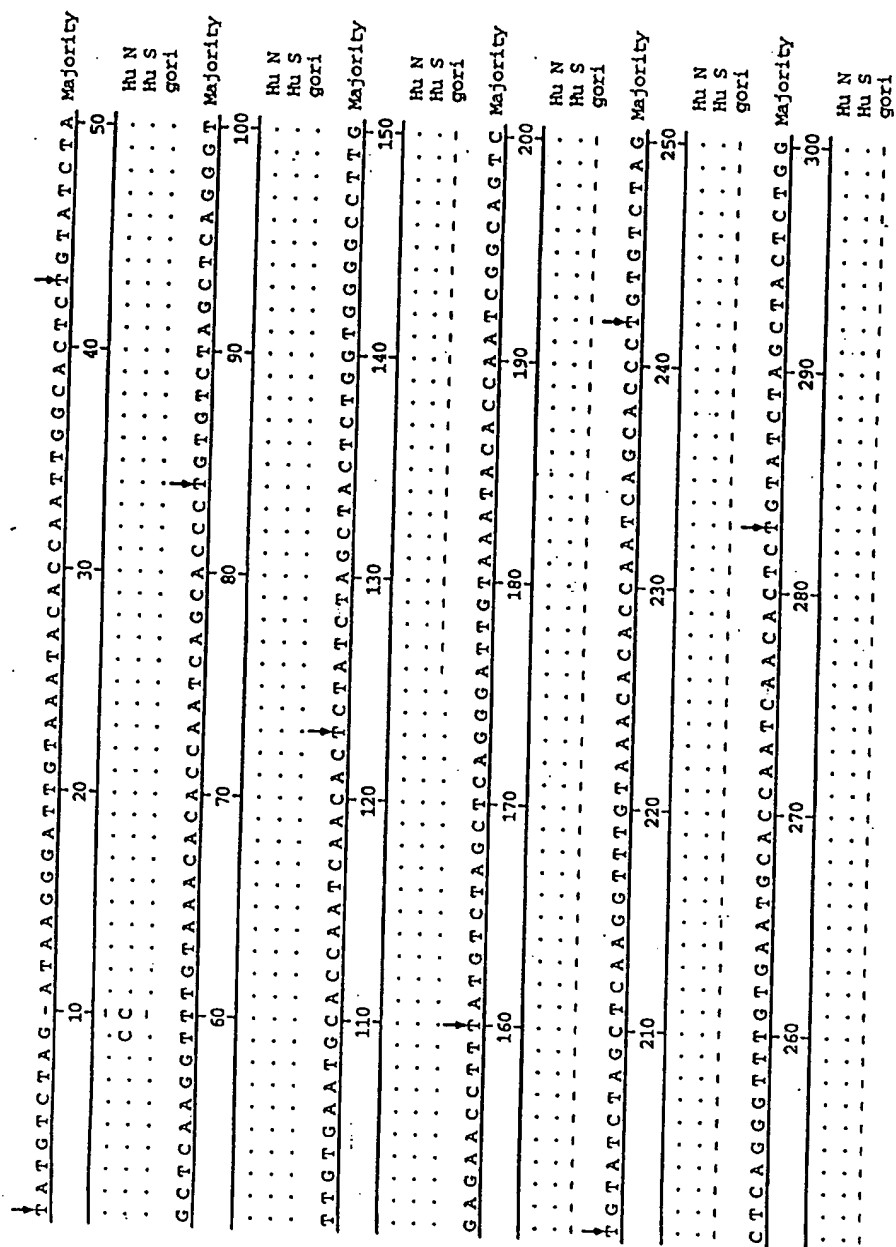


Figure 5A

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.	Hu N	
.	Hu S	
.	gori	
CTCGGC	AGTC	CTGT	ATCT	AGCT	CAAG	GTTT	TGTA	AAAC	ACAC	CAAC	Majority
360	370	380	390	400							
.	Hu N	
.	Hu S	
.	gori	
CTGTG	CTAGC	TCA								Majority	
410	420	430	440	450							
.	Hu N	
.	Hu S	
.	gori	
										Majority	
460	470	480	490	500							
GCTAC	TCTG	TGGG	ACTT	GGAG	AAC	CTTT	TGTG	TGG	AC	ACTCT	Hu N
										Hu S	
										gori	
CTAAT	CTGG	TGGG	AXGT	GGAG	AAC	CTTT	TGTG	TCTAG	CTCA	TGGATTG	Majority
510	520	530	540	550							
.	Hu N	
.	Hu S	
.	gori	
AAATG	CACCA	ATCA	GTG	CCCT	GTCA	AAAC	AGAC	CACT	GGG	CTCT	Majority
560	570	580	590	600							
.	Hu N	
.	Hu S	
.	gori	

Figure 5B

T C A G C A G G A T G T G G G T G G G C C C A G A T A A G A G A A T A A A A G C A G C C T G C C C G	610	620	630	640	650	Majority
.						
.						Hu N
.						Hu S
.						gori
A G C C A G C A G T G G C A A C C C G C T C G G G T C C C C T T C C A C A C T G T G G A A G C T T	660	670	680	690	700	Majority
.						
.						Hu N
.						Hu S
.						gori
G T T C T T T C G C T C T T T G C A A T A A T C T T G C T G C T G C T C A C T G T T T G G G T C T	710	720	730	740	750	Majority
.						
.						Hu N
.						Hu S
.						gori
A C A C T G C C C T T T A T G A G C T G T A A C G C T C A C C G C G A A G G T C T G C A G C T T C A C	760	770	780	790	800	Majority
.						
.						Hu N
.						Hu S
.						gori
T C T T G A A G C C A G C G A G A C C A C G A A C C C A C C G G G A G G A A C G A A C A C T C C A	810	820	830	840	850	Majority
.						
.						Hu N
.						Hu S
.						gori
G A G G C G C C G C C T T A A G A G C T G G A A C G T T C A C T G - T G A A G G T C T G C A G C T T	860	870	880	890	900	Majority
.						
.						Hu N
.						Hu S
.						gori

Figure 5C

C A C T C C T G A G C C A G C G A G A C C A C G A A C C C A T C A G A A G G A A G A A C T C C G A	910	920	930	940	950	Majority
.	Hu N
.	Hu S
.	gori
A C A C A T C C A A A C A T C A G A A C G A A C A A A C T C C A C A C A C G C C T T T A A G A	960	970	980	990	1000	Majority
.	Hu N
.	Hu S
.	gori
A C T G T A A C A C T C A C C A C G A G G T C C C - C G G C T T C A T T C T T G A A - G T C A G T	1010	1020	1030	1040	1050	Majority
.	Hu N
.	Hu S
.	gori
G A A C C A A G A A C C C A C C A A T T C C G G A C A C A G T A T G T C A G A A A C A A T A T G A	1060	1070	1080	1090	1100	Majority
.	Hu N
.	Hu S
.	gori
G T C A C T A A A T C A A T A T A C T T C T C A A C A A T T T C C A A C A G C C C T T G C A A T T A	1110	1120	1130	1140	1150	Majority
.	Hu N
.	Hu S
.	gori
A C T T G G C C A T G T G A C T G G T T G T G A	1160	1170				Majority
.	Hu N
.	Hu S
.	gori

Figure 5D

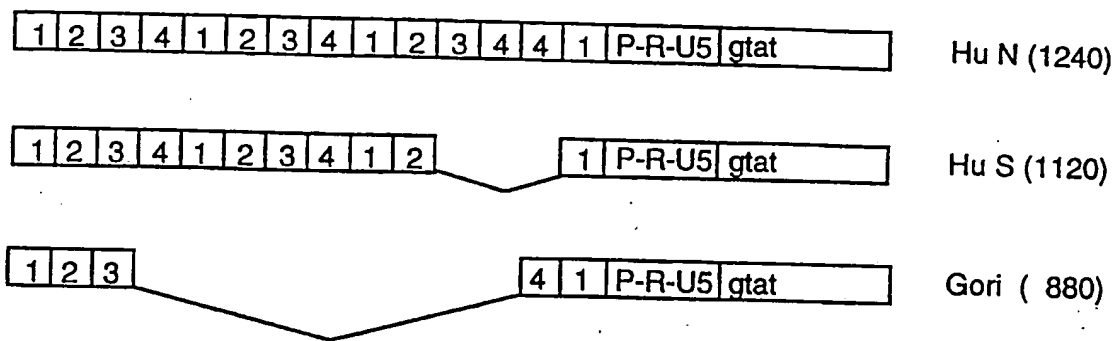


Figure 6

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Figure 7

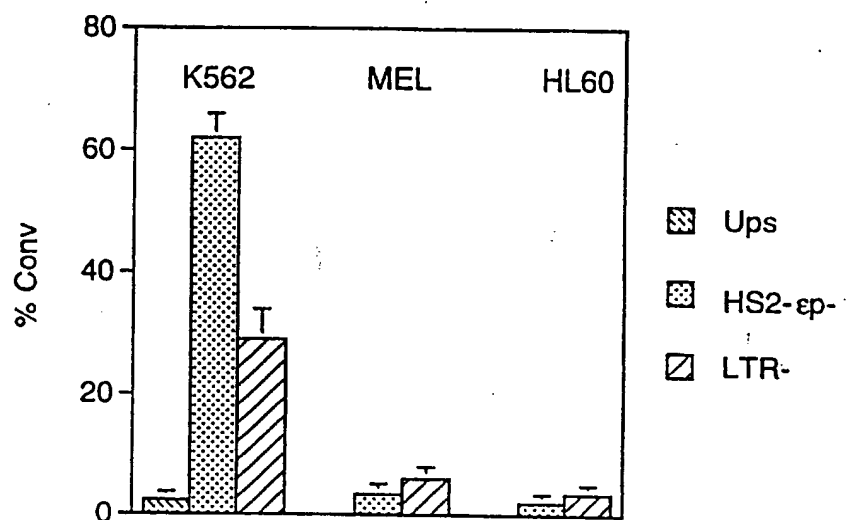


Figure 8

14/18

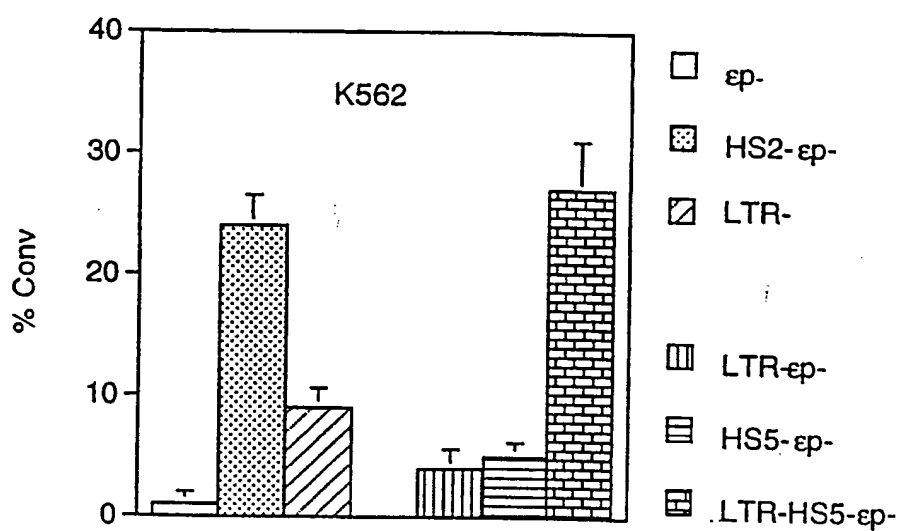


Figure 9

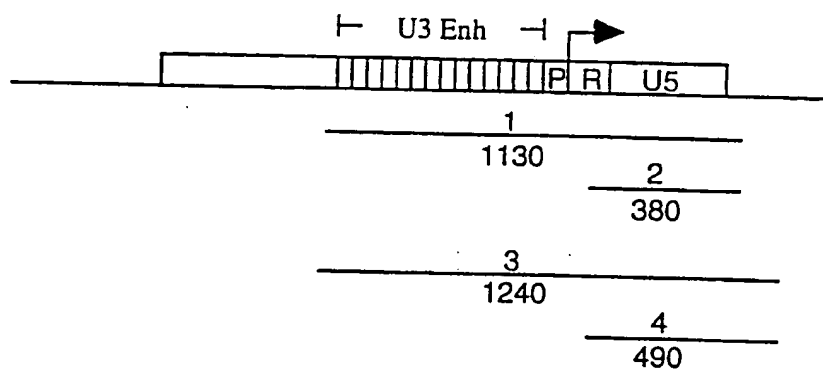
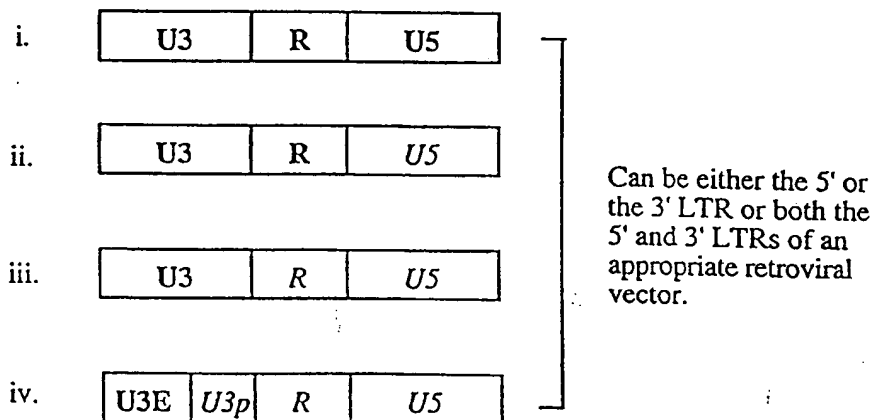


Figure 10

1. To replace the LTRs or their component U3, R and U5 regions of retroviral vectors designed for gene therapy of hereditary or acquired hematological diseases.



U3: the U3 enhancer and promoter of the 5'HS5 ERV-9 LTR

R: the R region of the 5'HS5 ERV-9 LTR

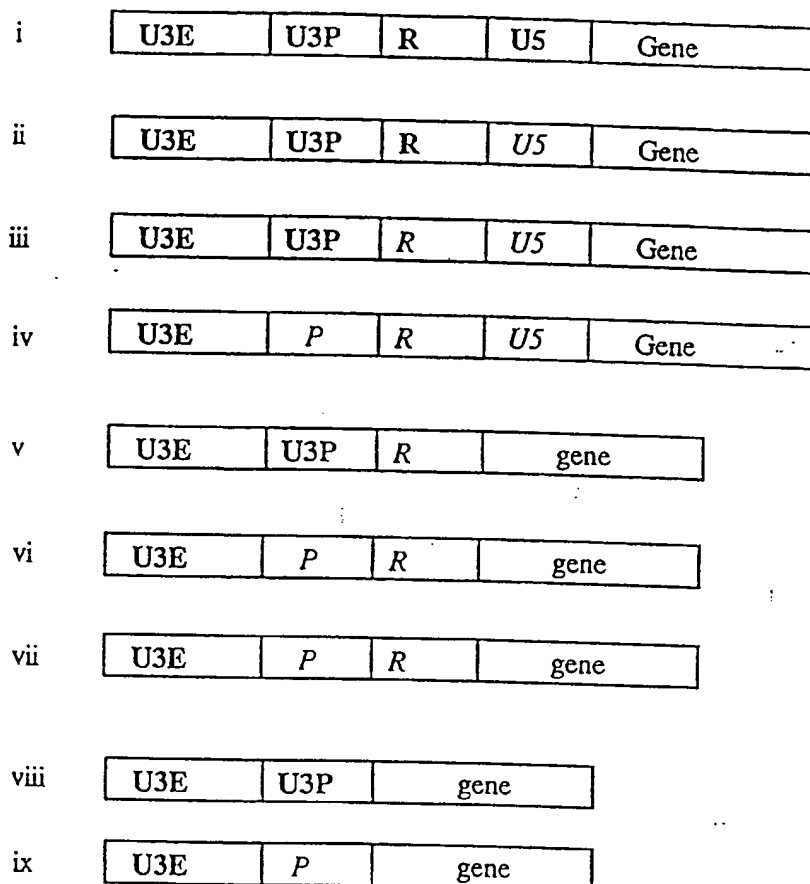
U5: the U5 region of the 5'HS5 ERV-9 LTR

U3E: the U3 enhancer of the 5'HS5 ERV-9 LTR

U3p, R and U5: the U3 promoter, R and U5 regions of appropriate non-5'HS5 ERV-9 LTRs.

Figure 11

2. To activate in hematopoietic cells the transcription of a cis-linked transgene spliced in either viral or non-viral vectors.



U3: the U3 enhancer and promoter of the 5'HS5 ERV-9 LTR

R: the R region of the 5'HS5 ERV-9 LTR

U5: the U5 region of the 5'HS5 ERV-9 LTR

U3E: the U3 enhancer of the 5'HS5 ERV-9 LTR

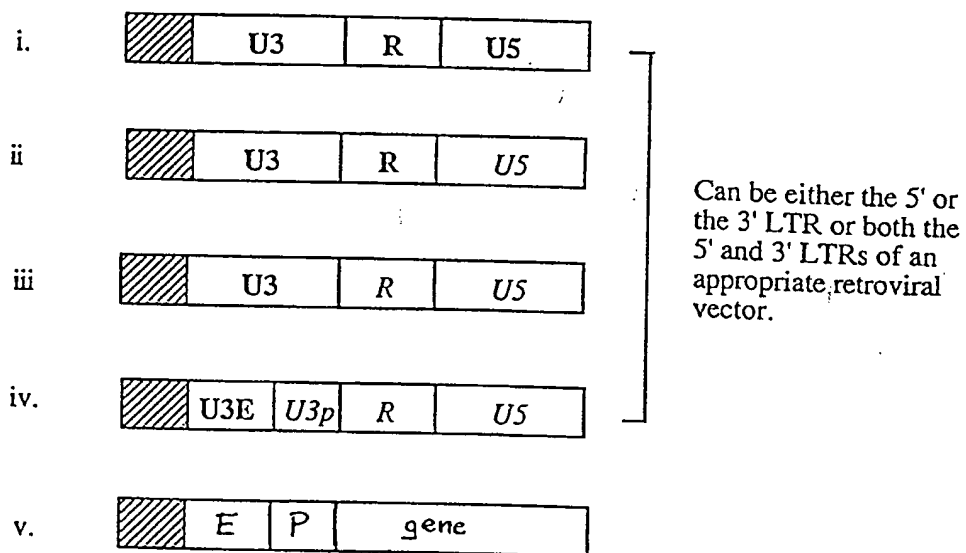
U3P: the U3 promoter of the 5'HS5 ERV-9 LTR

R and U5: the R and U5 regions of appropriate non-5'HS5 ERV-9 LTRs.

P: appropriate promoter other than the U3 promoter of the 5'HS5 ERV-9 LTR.

Figure 12

Design of the vectors:



Hatched box: the U3 insulator sequence.
 E and P: appropriate enhancer and promoter sequences.
 gene: appropriate transgene
 all other designations: the same as Figure 12

Figure 13

SEQUENCE LISTING

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<400> 7

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 ctctgtatct agctaactctg gtggggaagt ggagaacctt tgtgtctagc tcagggattg 180
 taaacgcacc aatcagcacc ctgtcaaaac agaccactgg gctctaccaaa tcagcaggat 240
 gtgggtgggg ccagataaga gaataaaagc aggttgccca agccagcagt ggcaacgtgc 300
 tcaggtcccc tcccacactg cggaagcttt gttctttcgc tctttgcaat aaatcttgct 360
 gctgtctact gtttgggtct acactgcctt tacgagctat aacgctcacc cgaaggctctg 420
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 acaaactcca cacacgcagc ctttaagaac tgtaaacactc accacgaggg tcccgcggct 660
 tcattcttga aagtcagtg aaccaagaac ctaccaattc ggacacagta tgtcagaaac 720
 aatatgagtc actaaatcaa tatacttctc aacaatttcc aacagccctt gcaattaact 780
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<211> 41

<212> DNA

<213> Homo sapiens

<400> 8

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<210> 9

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tgtctagctc aaggtttgta aacacaccaa tcagcaccct g 41

<210> 10

<211> 41

<212> DNA

<213> Homo sapiens

<400> 10

tatctagctc agggtttgtg aatgcaccaa tcaacactct g 41

<210> 11

<211> 37

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<213> Homo sapiens

<400> 11

tgtctagcta ctctggtggg gacgtggaga accttta 37

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<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: consensus
sequence

<400> 12

trtctagctc adggtttgtr aayrcaccaa tcagcactct g 41

<210> 13

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: consensus
sequence

<400> 13

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<210> 14

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: consensus

sequence

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41

<210> 15

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
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<400> 15

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<210> 16

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
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<210> 17

<211> 22

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<210> 18

<211> 26

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<400> 18

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<210> 19

<211> 25

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<400> 19

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25

<210> 20

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
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<400> 20

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25

<210> 21

<211> 26

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<400> 21

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26

<210> 22

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
oligonucleotide

<400> 22

gatggatcca atagattttt gtcattct

27

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/24646

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/85 C12N15/867

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>LONG OM ET AL: "Nucleotide sequence and transcriptional analysis of DNA upstream of hypersensitive site 4 in the human beta globin locus control region"</p> <p>BLOOD,</p> <p>vol. 86, no. 10, supplement 1,</p> <p>15 November 1995 (1995-11-15), page 472a</p> <p>XP000877143</p> <p>cited in the application</p> <p>abstract 1874</p> <p style="text-align: center;">— — — — — — / —</p>	1-8, 10-12, 35

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"a" document member of the same patent family

Date of the actual completion of the international search

7 March 2000

Date of mailing of the international search report

21/03/2000

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 Fax (+31-70) 340-3018

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/US 99/24646

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANDERSSSEN S ET AL: "Comparative analyses of LTRs of the ERV-H family of primate-specific retrovirus-like elements isolated from marmoset, african green monkey, and man" VIROLOGY, vol. 234, no. 1, 21 July 1997 (1997-07-21), pages 14-30, XP002132290 ORLANDO US figure 2	30,34
X,P	DATABASE GENBANK 'Online! Sequence AF064190 , 9 February 1999 (1999-02-09) LONG QM ET AL: "Homo sapiens beta-globin locus control region, 5' sequence" XP002132292 cited in the application compare nucleotides 2656-4493 with SEQ ID NO:1	1-8, 10-12,35
P,X	LONG Q ET AL: "A long terminal repeat of the human endogenous retrovirus ERV-9 is located in the 5' boundary area of the human beta-globin locus control region" GENOMICS, vol. 54, no. 3, 15 December 1998 (1998-12-15), pages 542-555, XP000884347 the whole document	1-8, 10-12, 16-26, 30-35
A	REIK A ET AL: "The locus control region is necessary for gene expression in the human beta-globin locus but not the maintenance of an open chromatin structure in erythroid cells" MOLECULAR AND CELLULAR BIOLOGY, vol. 18, no. 10, October 1998 (1998-10), pages 5992-6000, XP002132291 page 5098, right-hand column, last paragraph	1-12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/24646

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14, 15 and 28 and 29
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the vector.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.